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Macular Pigment and Diabetes Mellitus

Grainne Scanlon

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Grainne Scanlon. Dip Optom, BA Psychology, MPhil.

PhD Thesis

Technological University Dublin

Supervisors: Prof James Loughman

Dr Daniel McCartney

Dr John S Butler

School of Physics, Clinical and Optometric Sciences

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Abstract

Background/aims

The macula is a specialised part of the retina responsible for detailed central and colour vision. The carotenoids lutein, zeaxanthin and *meso*-zeaxanthin are uniquely concentrated in the inner and central layers of the primate macula, where they are collectively known as macular pigment (MP). Macular pigment confers potent antioxidant and anti-inflammatory effects in the eye. Many studies have suggested that these carotenoids are lower in age-related macular degeneration (AMD) and that increased levels of MP may confer protection against AMD, especially the late form of the disease. Research is now beginning to focus on MP optical density (MPOD) and carotenoid intake in diabetes mellitus, a condition which similar to AMD, is known to cause oxidative damage and inflammation in the retina.

Methods

The optical density of MP was measured in a group of participants with diabetes (Type 1 and Type 2) and findings compared with normal healthy controls. A comprehensive review was performed to investigate the putative causal mechanisms and metabolic perturbations associated with lower MP in diabetes. Surrogate biomarkers for the prediction of low MP in participants with Type 2 diabetes and older adults free of ocular pathology, including clinical [blood pressure], plasma [lipoproteins, inflammatory markers] and anthropometric [waist (cm), hip (cm), height (cm), weight (kg)] parameters, were investigated and identified as part of a large randomly selected sample from the Republic of Ireland (as part of The Irish Longitudinal Study on Ageing [TILDA]).

Results

The optical density of MP was lower among Type 2 diabetes subjects (0.33 ± 0.21) compared with Type 1 subjects (0.49 ± 0.23) and normal controls (0.48 ± 0.35) (p= 0.01). A comprehensive review of MP and diabetes, Type 2 diabetes, in particular, revealed that MP may become depleted through at least four possible causal mechanisms in this condition including overweight/obesity, dyslipidaemia, oxidative stress and inflammation. Research performed on the TILDA cohort confirmed that participants with Type 2 diabetes had significantly lower MPOD compared with non-diabetic controls (p=0.047). In-depth analysis on this Type 2 diabetes cohort revealed that MP was significantly lower in diabetes participants who were deficient in plasma vitamin D (<50 nmol/L) (p=0.006); who had a raised triglyceride (TG) over high-density lipoprotein (HDL) ratio (TG/HDL) [>1.74 mmol/L; p=0.039]; who had hypertension (p=0.043); who were current smokers (p=0.022); or who had cataracts (p=0.049). Among older adults who were free of ocular pathology (*i.e.* AMD, glaucoma, diabetes, pre-diabetes), MPOD was significantly lower among participants with an elevated waist circumference (WC) (p=0.034), those who had low plasma HDL (p=0.038), those with a raised plasma TG/HDL ratio (p=0.003) and those with a raised total cholesterol (TC) over HDL ratio (TC/HDL) (p=0.030).

Conclusion

Overall, our findings suggest that individuals with Type 2 diabetes have lower MP relative to healthy controls. The metabolic correlates associated with Type 2 diabetes, in particular, *i.e*. oxidative stress, inflammation, overweight/obesity and dyslipidaemia, may have important implications for MPOD in the retina. Surrogate biomarkers associated with lower MP in Type 2 diabetes include low plasma levels of

vitamin D (25(OH) D), dyslipidaemia (*i.e.* raised TG/HDL ratio), hypertension, cataracts and smoking. While an altered lipoprotein profile (*i.e.* low HDL, raised TG/HDL ratio, raised TC/HDL ratio), may affect the transport, uptake, and stabilisation of carotenoids in the retina of older adults free of ocular pathology, it appears that WC is a more robust predictor of lower MPOD in this patient cohort. However, its effect size appears to be small and therefore its clinical applicability is questionable.

Keywords: dyslipidaemia; high-density lipoprotein; macular pigment; triglycerides; vitamin D (25(OH) D), waist circumference.

Declaration

I certify that this thesis which I now submit for examination for my confirmation exam for my PhD, is entirely my own work and has not been taken from the work of others save and to the extent that such work has been cited and acknowledged within the text of my work.

This thesis was prepared according to the regulations for post graduate study by research of the Technological University Dublin and has not been submitted in whole or in part for an award in any other Institution or University.

The work reported on in this thesis conforms to the principals and requirements of the TU Dublin's guidelines for ethics in research.

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List of publications

Scanlon G, Butler JS, McCartney D, Loskutova, E, Kenny RA, Loughman J. Investigation of surrogate biomarkers associated with macular pigment status in a group of older Irish adults. Accepted for publication *Optom Vis Sci.* May 2020.

Scanlon G, Loughman J, Farrell D, McCartney D. A review of the putative causal mechanisms associated with lower macular pigment in diabetes mellitus. *Nutr Res Rev.* 2019; 32(2): 247-64.

Scanlon G, McCartney D, Butler JS, Loskutova E, Loughman J. Identification of surrogate biomarkers for the prediction of patients at risk of low macular pigment in Type 2 diabetes. *Curr Eye Res.* 2019; 44(12): 1369-80.

Scanlon G, Connell P, Ratzlaff M, Foerg B, McCartney D, Murphy A, O'Connor K, Loughman J. Macular pigment optical density is lower in type 2 diabetes, compared with type 1 diabetes and normal controls. *Retina.* 2015; 35(9):1808-16.

Abbreviations List

ADA	American Diabetes Association
AGE	Advanced Glycation End Product
AIF6	Activating Transcription Factor 6
AMD	Age Related Macular Degeneration
AMPK	Activated Protein Kinase;
ANOVA	Analysis of Variance
AREDS	Age Related Eye Disease Study
ARPE-19	Adult Retinal Pigment Epithelial Cell Line-19
AT 1	Angiotensin Type 1
ATC	Anatomic Therapeutic Classification
ATF 6	Activating Transcription Factor 6
β	Beta
BCO1	β-Carotene 15,15′ oxygenase-1
BCO2	β-carotene 9′,10′ oxygenase-2
BiP	Binding Immunoglobulin Protein;
BMI	Body Mass Index
BP	Blood Pressure
BM	Bruch's Membrane
Ca^{2+}	Calcium
CAREDS	Carotenoids in Age-Related Eye Disease Study
CAT	Catalase
CD36	Cluster of Differentiation 36
CETP	Cholesteryl Ester Transfer Protein
CH	Choriocapillaris

COX-2	Cyclooxygenase-2
CRP	C-Reactive Protein
c-HFP	Customised Heterochromatic Flicker Photometry
DAG	Diacylglycerol
DCCT	Diabetes Control and Complications Trials
DHA	Docosahexaenoic Acid
DiVFuSS	Diabetes Visual Function Supplement Study
DNA	De-Oxy-Ribonucleic Acid
DXA	Dual Energy X-Ray Absorptiometry
EAS	European Atherosclerosis Society
ECF	Extra Cellular Fluid
EDTRS	Early Treatment of Diabetic Retinopathy Study
EIU	Endoxin-Induced Uveitis
ELM	External Limiting Membrane
eNOS	Endothelial Nitric Oxide Synthase
EPA	Eicosapentanoic acid
ERG	Electroretinogram
ERK	Extra Cellular Receptor Kinase;
ESC	European Society of Cardiology
F	Female
FA	Fatty Acid
FAZ	Foveal Avascular Zone
FFA	Free Fatty Acid
FFQ	Food Frequency Questionnaire
FOXO 3α	Forkhead O Transcription Factor 3 α;

GABA	Gamma-Aminobutyric Acid
GCL	Ganglion Cell Layer
GFAT	Glutamine Fructose-6 Phosphate Amidotransferase
GLUT-1	Glucose Transporter-1
GPX	Glutathione Peroxidase
GSH	Glutathione
GSTP1	Glutathione S-Transferase P1
HbA1c	Glycated Haemoglobin
HDL	High Density Lipoprotein
HFP	Heterochromatic Flicker Photometry
HPLC	High Performance Liquid Chromatography
hs-CRP	High Sensitivity C-Reactive Protein
HSL	Hormone Sensitive Lipase
8-OHdG	8-hydroxy-2^1 – deoxyguanosin
ICAM-1	Inflammatory Intercellular Adhesion Molecule-1
IGF-1	Insulin Growth factor-1
IKK-β	Inhibitor of Nuclear Factor Kappa-B by Kinase subunit beta
IL-1	Interleukin-1
IL-1β	Interleukin -1 beta
ILM	Inner Limiting Membrane
IN	Inferior Nasal
INL	Inner Nuclear Layer
INOS	Inducible Nitric Oxide Synthase;
IPL	Inner Plexiform Layer
IS	Inner Segment

IT	Inferior Temporal
IQ	Intelligence quotient
JNK-1	c-Jun-N-Terminal Kinase-1
K^+	Potassium
kcal	kilo-Calorie
Kg/m^2	Kilogram per metre squared
LC-PUFA	Long Chain-Poly Unsaturated Fatty Acid
LDL	Low Density Lipoprotein
LPL	Lipoprotein Lipase
M	Male
MDA	Malondialdehyde
Mf-ERG	Multifocal-electroretinogram
Mg	Milligram
Mg/day	Microgram per Day
µg/l	Microgram per Litre
mg/dl	Milligram per decilitre
mmol/L	Milli Moles per Litre
Mn-SOD	Manganese Super Oxide Dismutase
MP	Macular Pigment
MPOD	Macular Pigment Optical Density
NADPH	Nicotinamide Adenine Dinucleotide Phosphate
NF-kβ	Nuclear Factor –kappa β
NFL	Nerve Fibre Layer
nmol/L	Nano Moles per Litre
NO	Nitric Oxide

OCT	Optical Coherence Tomography
OCTA	Optical Coherence Tomography Angiography
OLM	Outer Limiting Membrane
ONL	Outer Nuclear Layer
OPL	Outer Plexiform Layer
OS	Outer Segment
P-AKT	Phosphorylated Serine/Threonine Kinase.
PEDF	Pigment-Epithelium Derived Factor
PERK	Protein kinase RNA-like ER kinase
PKC	Protein Kinase C
PRDF	Pigment Epithelium Derived Factor
PUFA	Polyunsaturated Fatty Acid
RAGE	Receptor for Advanced Glycation End Product
RAS	Renin Angiotensin System
RCT	Randomised Control Trial
ROS	Reactive Oxygen Species
RPE	Retinal Pigment Epithelium
SD	Standard Deviation
SES	Socio Economic Status
SN	Superior Nasal
SOD	Super Oxide Dismutase
SR –B1	Scavenger Receptor Class B Type 1
SST	Somatostatin
ST	Superior Temporal
STARD	StAR-related lipid transfer protein

STAT3	Signal Transducer Activator Transcription 3
STZ	Streptozotocin
TC	Total Cholesterol
TG	Triglyceride
TILDA	The Irish Longitudinal Study of Ageing
TNF-α	Tumor-Necrosis-Alpha
UKPDS	United Kingdom Prospective Diabetes Study
UV	Ultra-Violet
VA	Visual Acuity
VDR	Vitamin D Receptor
VEGF	Vascular Endothelial Growth Factor
VLDL	Very Low Density Lipoprotein
WC	Waist Circumference
WHAM	Wisconsin Hypo Alpha Mutant
WHpR	Waist-to-Height-Ratio
WHtR	Waist-to-Hip-Ratio
WHO	World Health Organisation

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1. INTRODUCTION

1.1 Background

The hydroxyl-carotenoids lutein, zeaxanthin and the retinal metabolite of lutein, *meso*-zeaxanthin, concentrate at the macula where they are collectively known as macular pigment (MP).[1] The selective accumulation of only three specific dietary carotenoids, to the exclusion of all other carotenoids in nature, suggests an exquisite biological function at the site of best vision and indicates a role that is uniquely suited to this anatomic location. It has been shown that MP can influence the quality of visual performance through the selective absorption of short-wavelength light before photoreceptor light capture.[2] Macular pigment also protects the retina from photo-oxidative damage by means of its optical filtration and/or antioxidant properties.[2, 3] Evidence is now emerging that these carotenoids exhibit a neuroprotective and anti-inflammatory function in the retina.[4] While there is a growing body of evidence to suggest that MP is lower in patients with age-related macular degeneration (AMD), and that increased levels of MP may confer protection against this disorder,[3, 5] only a small number of studies have focused on carotenoid intake and MP optical density (MPOD) in diabetes mellitus.[6, 7] Diabetic retinopathy is one of the primary microvascular complications of diabetes and the leading cause of blindness in the working-age population.[8] There is strong evidence to suggest that oxidative stress [9] and its associated inflammation [10] play an important role in the development of diabetic retinopathy. Macular pigment may, therefore, through its antioxidant, anti-inflammatory and/or pre-receptoral filtration properties; [2-4] confer protection against diabetic eye disease. This is the focus of the current research.

1.2 Rationale

The condition diabetes mellitus is explored in detail. Whilst diabetes is characterised by having higher than normal blood glucose levels, the pathogenesis and development of the two main forms of the disease, Type 1 and Type 2 diabetes, differ, and may, therefore, have unique and independent relationships with MP. While there is no cure for diabetic retinopathy, new approaches are now being taken to understand the pathophysiology of the disease to help improve its detection, prevention, and treatment. Current treatment paradigms for diabetic retinopathy include laser photocoagulation,[11] intravitreal injections,[12] and vitreoretinal surgery,[13] all of which focus on the advanced form of the disease, often after permanent damage has occurred. Newer treatments which are preventative in nature or which address early pathological changes are therefore highly desirable. Research is now beginning to examine the possible beneficial effects of dietary supplemental lutein, zeaxanthin, and *meso*-zeaxanthin in protecting ocular tissues and cells, including retinal neurons, in the hope that these phytochemicals may retard disease progression, a concept explored in section one.[14, 15]

An initial evaluation of the available evidence suggested that MP appears to be generally lower in populations with diabetes compared with healthy controls,[6] in particular amongst patients with Type 2 diabetes.[7] A comprehensive review of the available evidence pertaining to the relationship between diabetes mellitus and MPOD was undertaken, therefore, with the primary focus to explore the putative causal mechanisms and metabolic perturbations which might explain any associations between diabetes, (Type 2 diabetes in particular) and MP status. Metabolic correlates associated with Type 2 diabetes include overweight/obesity,[16, 17] dyslipidaemia,[18, 19]

oxidative stress,[9] and inflammation;[10] mechanisms which may have both independent and synergistic relationships with MP, a concept which is explored in detail in the review.

In addition to the review evidence, a body of experimental work was also designed and executed to; a) explore MP levels in a group of participants with diabetes mellitus (Type 1 & Type 2) relative to normal healthy controls; b) explore this concept further in Type 2 diabetes participants derived from a large representative population of older adults as part of the Irish Longitudinal Study of Aging (TILDA), with specific focus on the identification of surrogate biomarkers for the prediction of low MP in these Type 2 diabetes subjects; and c) identify proxy biomarkers associated with lower MP in a group of older Irish adults in TILDA who are free of ocular pathology (*i.e.* AMD, glaucoma, diabetes, pre-diabetes).

1.3 Research benefits

Macular pigment has several important functions in the eye including blue light filtration and potent antioxidant activities.[2, 3] More recently, research has suggested that MP also has neuroprotective and anti-inflammatory effects in the eye.[4] Diabetic retinopathy is the leading cause of blindness in the working-age population,[8] and chronic disease progression is believed to be linked to the interaction between inflammation [10] and oxidative stress.[9] There is a growing body of evidence to suggest that increased levels of MP may confer protection in AMD,[3, 5] and there is also preliminary evidence from animal studies to suggest a similar protective effect in diabetes.[14, 20] In humans, a recent randomised control trial (RCT) in this area has also yielded promising data which suggests that higher MP levels may prevent or retard the progression of diabetic retinopathy.[21]

Optometrists and eye care practitioners can play an important role in preventative eye care. At a community level, screening for lutein, zeaxanthin and MP levels in the macula would help identify patients at risk of low MPOD and stratify those patients requiring appropriate dietary intervention and/or additional supplementation. The density of MP can be augmented by eating foods rich in these carotenoids (*i.e.* green leafy vegetables, fruits, and legumes).[22] Supplements rich in these phytonutrients are also widely available. The MPOD can be measured *in vivo* [23] and represents a long-term measure of dietary carotenoid accumulation over months to years. However, MPOD is not routinely measured in clinical practice. Consequently, there may be value in exploring commonly measured surrogate biomarkers for the prediction of patients at risk of low MP, given that higher levels of MP may be beneficial to the retina. Clinical benefits may be realised by using these proxies to identify patients at increased risk of MPOD depletion (*i.e.* patients with Type 2 diabetes, older adults free of ocular pathology), and the use of early intervention protocols to address these MPOD deficits.

Given the myriad of mechanisms which may contribute to MPOD depletion in Type 2 diabetes, and the ocular risks putatively associated with lower MPOD in diabetes, lifestyle modification which involves a comprehensive dietary approach is a logical first step to optimise MPOD status and ocular health outcomes. *De novo* synthesis of MP is not possible in humans, therefore, the importance of diet cannot be overemphasised in the management of diabetic eye disease risk. Nutritional advice on the inclusion of foods rich in the carotenoids lutein and zeaxanthin (*i.e.* fruit, vegetables, legumes), and other dietary strategies to optimise lipoprotein status (*i.e.* triglycerides (TGs), high-density-lipoprotein (HDL), low-density-lipoprotein (LDL))

may, therefore, have long-term clinical impacts on disease prevention and progression. Dietary advice and lifestyle modifications which are also applicable to older adults identified with MP deficits.

Although the long-term benefits of maintaining high MP density during life are not yet known, there is the possibility that a more robust MP status over the full life course may retard disease progression in later years. Research has shown that neuroretinal changes can occur long before retinal signs of pathology become visible (*i.e.* micro-aneurysms, dot and blot haemorrhages in diabetic retinopathy). Therefore, increased awareness of the ocular risks associated with certain metabolic disturbances, (*i.e.* increased visceral fat, dyslipidaemia) and lifestyle choices (*i.e.* tobacco use) may yield behavioural changes which ultimately confer long-term visual health benefits, not only in patients with diabetes mellitus but also in older adults free of ocular pathology.

2. THE RETINA

2.1 Introduction

Diabetes mellitus is a group of metabolic disorders characterised by hyperglycaemia, arising from defects in either insulin secretion, insulin action or both.[24] Diabetic retinopathy is the most frequent microvascular complication of the disease and the most common cause of blindness in the working-age population.[25] The main reasons for vision loss are diabetic macular oedema and proliferative diabetic retinopathy. The likelihood of developing retinopathy is related to the duration of disease, hyperglycaemia, hypertension and possibly dyslipidaemia.[26] Type 1 and Type 2 diabetes are the two main forms of the disease.[27] Type 2 diabetes has an insidious onset and can go unnoticed for many years. As a result, patients may already have diabetic retinopathy at the time of diagnosis.[25] Conversely, Type 1 diabetes patients are diagnosed promptly and typically do not develop retinopathy until years after the diagnosis is made. Approximately 60% of Type 2 diabetes patients and nearly all Type 1 patients, however, will show some signs of retinopathy 20 years after disease diagnosis.[25] Although the incidence and progression of the potentially blinding complications of retinopathy can be reduced with tighter glucose control,[28] the fundamental causes of diabetic retinopathy remain uncertain. The unique anatomy and physiology of the retina may even make it uniquely susceptible to the metabolic stresses of diabetes.[29] In this regard, an understanding of the anatomy and physiology of the normal retina may give us better insight into what can go wrong in diabetes, and how these pathological mechanisms can be averted. The pathological sequelae of diabetes which are relevant to ocular health will be explored in this chapter.

2.2 The retina

The retina is the light-sensitive layer that extends over the inner surface of the back of the eyeball, lying in contact with the vitreous matter internally and with the vascular layer, the choroid, externally. The retina is a transparent layer of neural tissue. The gross anatomy of the retina is divided into the outer pigmented epithelial layer and the inner neural sensory layers. These layers form a functional unit essential for vision.

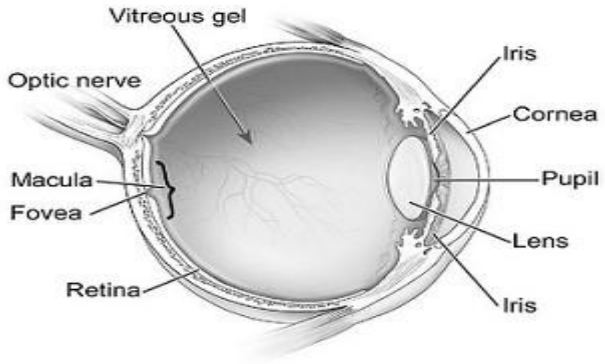


Figure 2.1: Schematic drawing of the human eye (Adapted from Morello [30]).

Normal vision depends on cell to cell communication between the neuronal, glial, microglial, vascular, and pigmented epithelial cells of the retina.[29] The major functions of the retina are to capture photons of light, convert this photochemical energy into electrical energy, integrate the resulting action potentials and transmit them to higher brain centres where they are interpreted into recognisable images.[29] The neural components of the eye are an extension of the forebrain and thus form part of the central nervous system.

The structure of the retina consists of ten distinct layers, of which there are two synaptic layers. The lamellar cellular architecture of the retina (Figure 2.2), has alternating layers of neurons (outer and inner nuclear layers and ganglion cell layer)

interposed with two plexiform layers where neurons communicate and where visual signals must synapse as they emerge from the rods and cones on their way to the optic nerve.

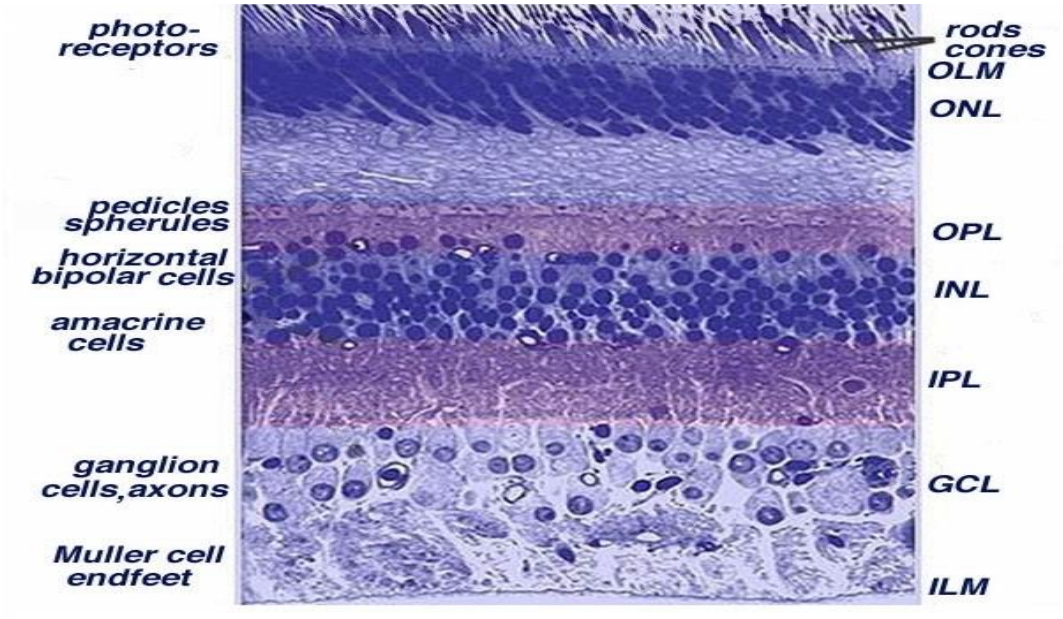


Figure 2.2: Light micrograph of a vertical section through central human retina (Adapted from Kolb et al, [31]).

Abbreviations: OLM-outer limiting membrane; ONL-outer nuclear layer; OPL-outer plexiform layer; INL-inner nuclear layer; IPL-inner plexiform layer; GCL-ganglion cell layer; ILM-inner limiting membrane.

The anatomy of the retina in Figure 2.3 is described in relation to optical coherence tomography (OCT), a technique currently used in the diagnosis and follow-up of diabetic retinopathy. It allows the multiple layers of the retina and choroid to be seen according to the classification laid down by the international panel of experts in vitreoretinal diseases.[32]

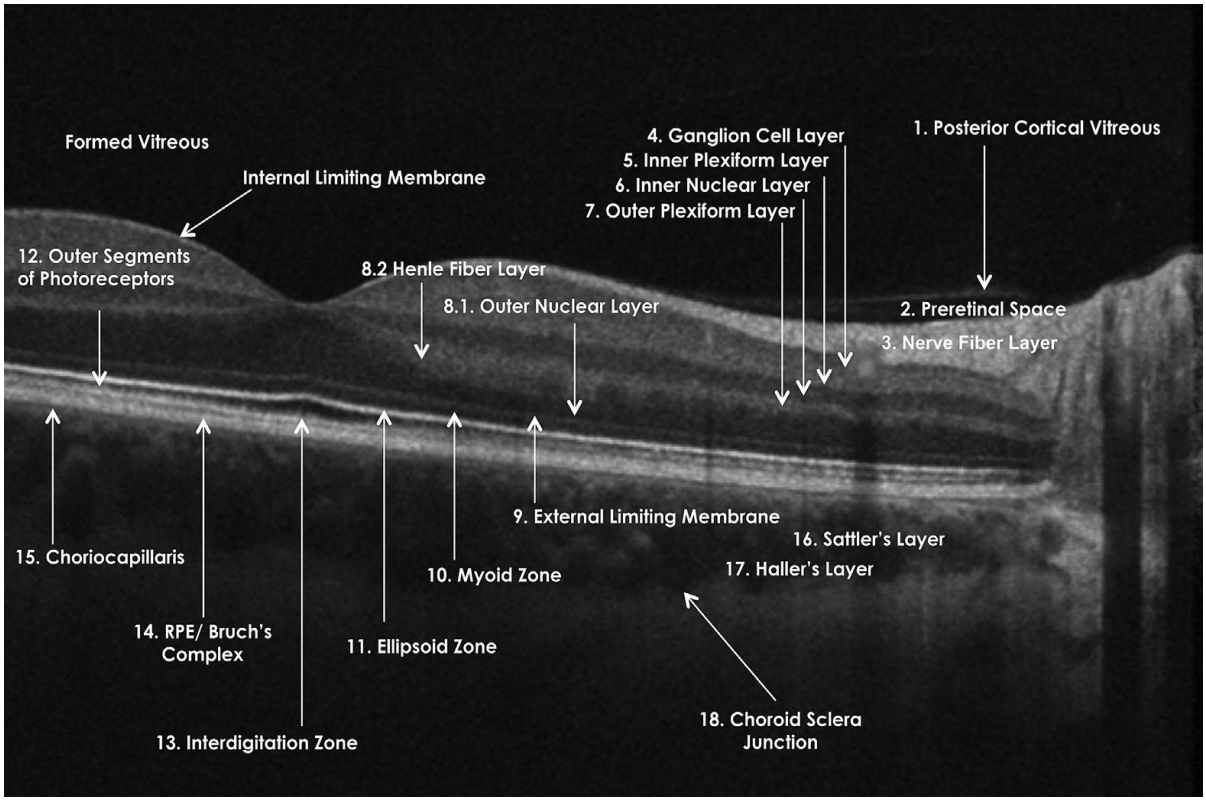


Figure 2.3: Nomenclature for normal anatomic landmarks seen on spectral-domain OCT images proposed and adopted by the International Nomenclature for OCT Panel. Healthy retina imaged using Zeiss Cirrus.[32]

Abbreviation: RPE = retinal pigment epithelium.

2.3 The macula

The macula represents the central part of the retina which is specialised for high spatial resolution and colour vision. The term macula derives from the presence of xanthophyll pigments, lutein, zeaxanthin, and *meso*-zeaxanthin, appearing as a yellow spot (macula lutea) when viewed in red-free light.[33] The macula is approximately 5.85 mm in diameter and is centered about 4 mm temporal and 0.8 mm inferior to the centre of the optic disc.[34] Based on microscopic anatomy, the macular area can be subdivided into several zones: the macula lutea (~5.85 mm diameter), fovea centralis (~1.85 mm diameter) and the foveola (~0.35 mm diameter) [35] (Figure. 2.4).

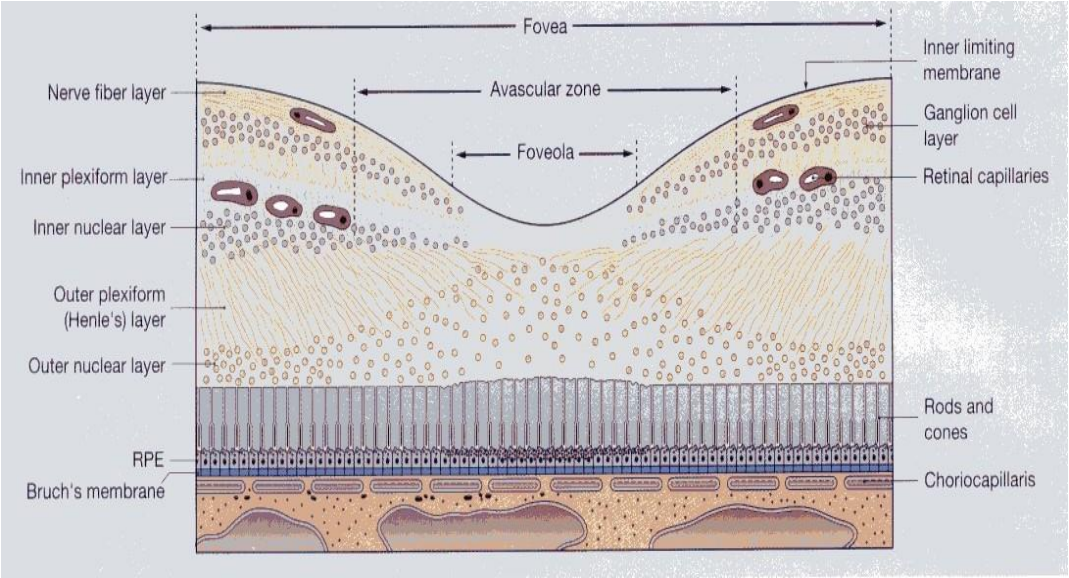


Figure 2.4: Schematic drawing of human foveolar (Adapted from Kanski et al,[36]).

The macular area can also be described and divided in terms of its different areas: the fovea containing the foveola; the parafovea and the perifovea [37] (Figure. 2.5). The foveola is surrounded by a wide ring zone where the ganglion cell layer, inner nuclear layer and outer plexiform layer of Henle are thickest. This is called the parafoveal area.[38] This area has a relatively low density of retinal vessels and below-average spatial density of rods.[39] The perifovea circumscribes the parafovea. The high rod: cone ratio and the high density of the retinal vasculature are features common to this area.[39]

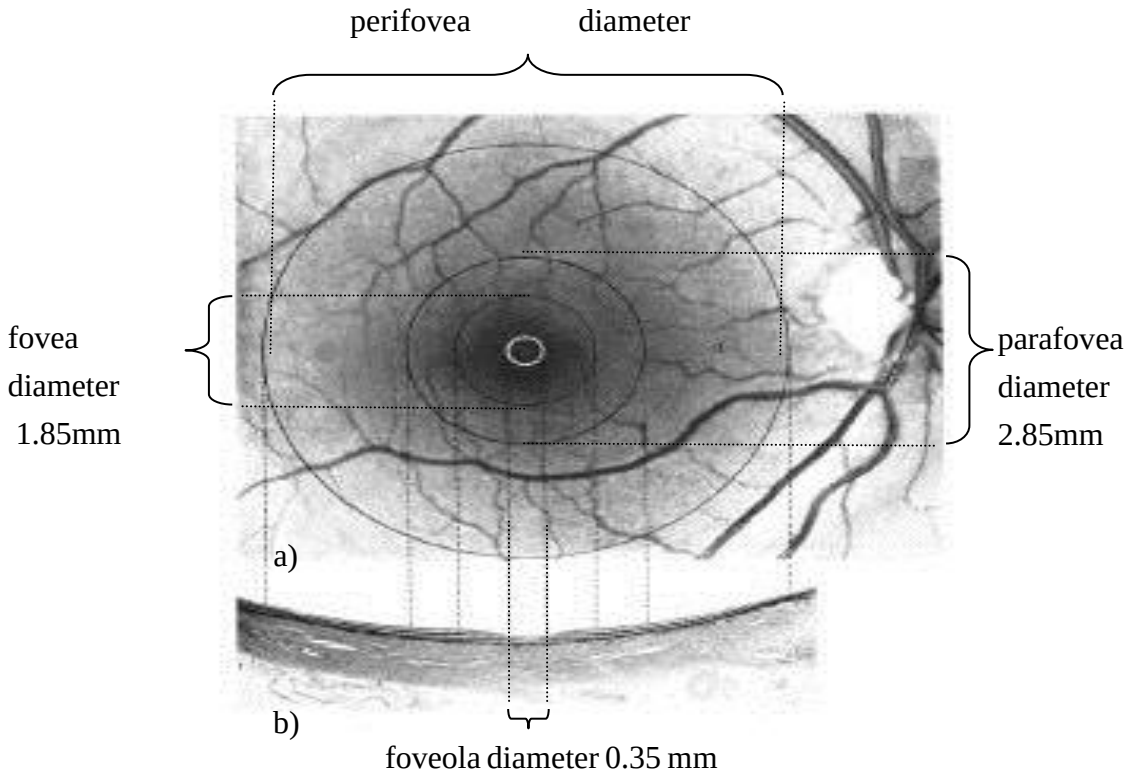


Figure 2.5: An anatomical view of the macula region as viewed through cross-section (Adapted from Thibos et al,[37]).

2.3.1 Specialisation of the macula

The central retina has evolved to enable us to see better *i.e.* to resolve fine spatial detail and allow colour perception. This is possible because of the unique structure of the macula which includes close packing and elongation of photoreceptors (especially cones), 'midget' circuitry which dominates the macular region, and refinement and specialisation of the central retinal vasculature.[40] The fovea accounts for almost all of our useful photopic vision, even though it comprises less than 4% of the total retinal area. The fovea has the highest cone density and the central 100 μm of the foveola contains only red and green cones.[39] Blue cone density is highest in a zone between 100 and 300 μm from the centre of the fovea, and the foveola is entirely rod free.[39]

An important factor contributing to foveal morphology is the predominance of a 'midget' circuitry, in which the majority of bipolar cells are 'midget bipolar cells' which each connects to a single cone in the outer nuclear layer and a single ganglion cell in the ganglion cell layer.[40] These 'midget' pathways are distinctive in the absence of convergence of photoreceptor signals onto bipolar and ganglion cells.[41] Conversely, 'parasol' cell circuits in the periphery are converging, and a single parasol ganglion cell receives input from multiple 'diffuse' bipolar cells and numerous receptors in the outer nuclear layer.[40] The foveola coincides with an area which is devoid of retinal blood vessels, called the 'foveal avascular zone' (FAZ). Most layers of the sensory retina are displaced sideways at the foveola to create the foveal pit, thus, photoreceptors have unimpeded stimulation from light forming the retinal image (Figure 2.6). This anatomic specialisation serves to maximise visual function by removing angioscotomas from the visual image at fixation.[42] Outside the fovea, rod photoreceptors dominate and second (horizontal, bipolar and amacrine cells) and third (ganglion cells) order neurons are present.

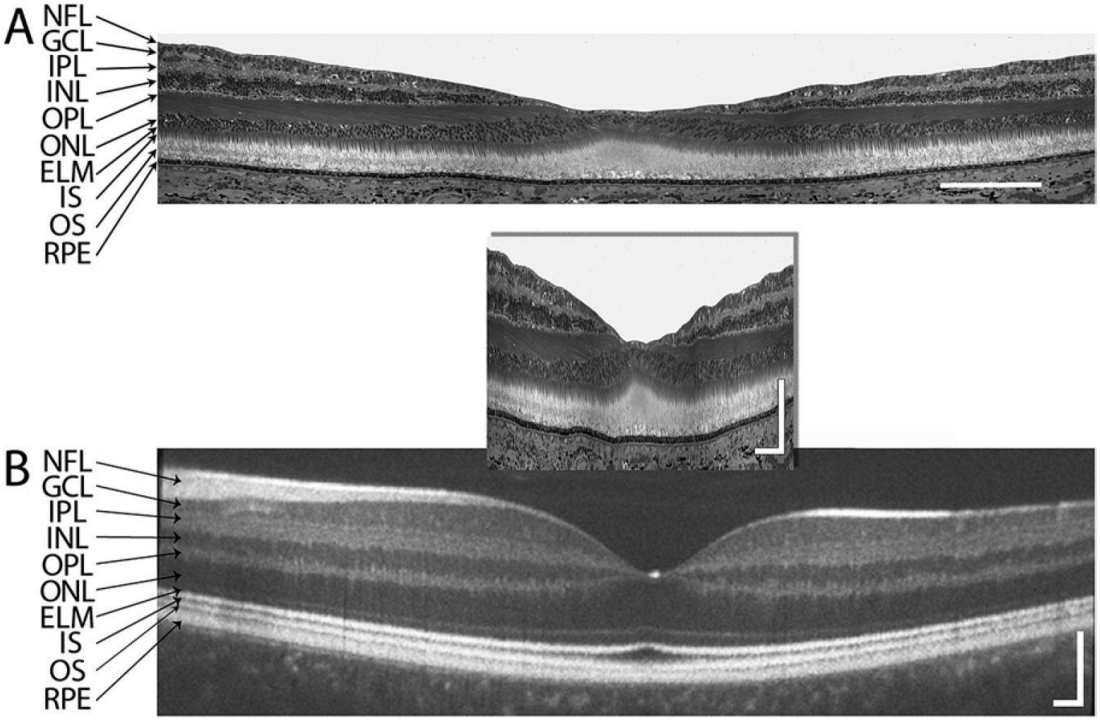


Figure 2.6: A comparison of histological and OCT images of the cross-sectional appearance of the human retina, at the fovea centralis (Adapted from Provis et al,[40]).

Abbreviations: NFL-nerve fibre layer; GCL-ganglion cell layer; IPL-inner plexiform layer; INL-inner nuclear layer; OPL-outer plexiform layer; ONL-outer nuclear layer; ELM-external limiting membrane; IS-inner segment; OS-outer segment; RPE-retinal pigment epithelium.

2.4 Macular pigment

Although MP is found throughout the tissue of the eye, it is concentrated in the macula lutea region of the retina. The primary localisation of human MP is in the inner retinal layers, in the fibres of Henle of the fovea, and parafoveally MP is located in the inner and outer plexiform layers.[43] The unique distribution of MP within the retina implies that these carotenoids play an important role in vision and macular health, and are

biologically relevant to the eye.[44, 45] Carotenoids lutein, zeaxanthin and *meso*-zeaxanthin are collectively referred to as MP.[46] *De novo* synthesis of MP is not possible in humans, therefore, must be obtained from dietary sources. Current studies indicate an ability to influence concentrations of carotenoids in MP by increasing concentrations ingested from foods [17, 47] and/or supplements.[48] Individual responses to dietary intake and/or supplementation, however, is quite variable and may change with time. Investigators have examined and identified possible predictors of MPOD, and found associations with age, [49, 50] sex,[51] overweight/obesity status,[52-54] and smoking,[52] among others, and will be discussed herein.

Age

Several lines of evidence suggest that MP may play a protective role against AMD, however, the association between age and MPOD remains unclear. Some studies have demonstrated a decline in MPOD with age, [49, 55, 56] while others have shown an increase [57, 58] and yet other studies have shown no such relationship.[59, 60] Research reporting on the association between age and MP is variable possibly due to differing methodologies being used to measure MPOD, differences in sample sizes and/or inconsistencies in age ranges. One recent study demonstrated that MPOD appeared to increase during adulthood (peak at 45-50 years) followed by a gradual reduction after 60 years.[61] This study postulated that if these findings can be confirmed in larger studies then it would be important to recommend supplementation, using lutein and zeaxanthin, in older populations as prophylaxis against age-related maculopathy.[61] Longitudinal data in larger cohorts, however, is required to satisfactorily investigate the relationship between the optical density of this pigment and age.

Gender

Gender differences in MPOD have also been reported.[16, 17] Hammond et al.[51] found that males had an average of 38% more MP than females and suggested that lutein might accumulate in the retina more readily in men than in women. While there was a positive correlation between plasma carotenoids and the density of MP for both sexes the relationship was stronger for men (males r=0.62, females r=0.3).[51] Another study found a significant negative correlation between adipose tissue lutein concentrations and MP for women and a significant positive correlation was found for men.[17] These researchers suggested that sex differences in lutein metabolism may be an important factor in tissue interactions and in determining MP density,[17] and that adipose tissue may compete for carotenoids more effectively in women than men. Not all research, however, supports these findings. A relative lack of MP was associated with adiposity in men, and these researchers suggested that this link might underlie the association between body fat and risk for AMD progression in males.[16]

Smoking

Several investigations have reported an association between cigarette smoking and MP.[49, 62 55, 63] Hammond et al.[64] compared MP measurements in participants who were current smokers compared with non-smokers, matched for age, sex, dietary patterns and overall pigmentation. Tobacco users had significantly lower MP (MPOD 0.16) compared with the controls (MPOD 0.34). Nolan et al.[52] reported not only a significant difference in MP between current and non-smokers but also an inverse dose-response relationship between MP and the number of cigarettes smoked per day. Research suggests that current smoking may lead to lower MP levels in the eye as smoking is associated with increased oxidative stress, not only through the increased systemic

production of free radicals but also though the weakening of the antioxidant system and/or poor diet.[65] Of interest, cigarette smoking is an established and important modifiable risk factor for AMD.[66] It is possible that the lack of macular carotenoids among smokers may shift the oxidant/antioxidant balance in favour of AMD.

Overweight/obesity

Studies have reported an inverse relationship between overweight/obesity and MPOD [16, 54] with the observed relationships largely attributable to participants with a BMI > 29 kg/m^2.[54] Although BMI measures do not provide a precise measure of adiposity it is likely that higher BMI levels are reflective of excess adipose tissue. Lutein, zeaxanthin and *meso*-zeaxanthin, the major components of MP, are fat-soluble pigments and adipose tissue is a major storage site of the macular carotenoids.[67] As body weight increases, it is suggested that more dietary xanthophyll carotenoids will be absorbed into adipocyte, thereby, making these carotenoids less available to the macula.[68] Also, increased adipose tissue, visceral fat, in particular, is associated with a state of chronic low-grade inflammation, which in turn leads to increased oxidative stress,[69] factors which may exert an even greater demand on antioxidant defences within the body, including MPOD.

Concentrations of carotenoids in the macular are highly variable. Apart from dietary intake, age, gender, current smoking and overweight/obesity, MP levels may also be influenced by other factors including the effective absorption of these phytonutrients,[70] competition among carotenoids for absorption, [17] dyslipidaemia, [18, 71] metabolic status, [6] and genetic variation.[72] Macular pigment will be discussed in more detail in chapter four.

2.5 Cell types in the retina

The retinal tissue contains both neuronal and non-neuronal elements which work together to enable vision and maintain retinal homeostasis. The five major cell types in the retina perform sensory, nutritional, regulatory and immunomodulatory functions.

2.5.1 The neurons

The neurons (photoreceptors, bipolar, horizontal, amacrine and ganglion cells) perform sensory functions and define colour perception, spatial resolution and contrast discrimination.[73] The laminated organisation of the retina generates two streams of visual information: a main or vertical pathway, from the photoreceptors to bipolar cells and from bipolar cells to ganglion cells; and a secondary lateral pathway, comprising local feedback circuits from horizontal cells back to photoreceptors,[74] and from amacrine cells back to bipolar cells.[75] Neurons mediate phototransduction and modulate and convey nerve impulses which are ultimately transmitted to the brain through the axons of the ganglion cells which comprise the nerve fibre layer and the optic nerve.[76] The distribution and variable density of photoreceptors and ganglion cells across the retina, the differential light sensitivity of photoreceptors and the convergence of information from the extra-foveal retina mean that a hierarchy exists in the architecture of retinal processing and that foveal information is given higher priority.[2] This hierarchy continues back to the striate cortex, where a high percentage of cortical cells are dedicated to foveal information [2] (*i.e.* cortical magnification).The fovea is particularly important for functional vision and blindness results when this area is lost to disease. The concept of neurodegeneration as an early component of diabetic retinopathy and the potential neuroprotective effects of MP in preserving the

integrity of the neural retina will be explored in more detail in chapters three and four.

2.5.2 Retinal glial cells

Three main types of glial cells are found in the mammalian retina which serves to maintain retinal homeostasis. These include macroglial retinal cells (Muller cells and astrocytes) and microglial cells. Glial cells exhibit distinct morphological and antigenic characteristics which not only provide structural support for the retina but which are also involved in metabolism, phagocytosis of neuronal debris and the release of certain trophic factors and neurotransmitters (reviewed by Vecino et al,[77]). Glial cells have intrinsic signalling systems which can spread in the form of calcium ion (Ca^{2+}) waves, and these are correlated with glutamate release.[77] This signalling process plays an important role in immunity, angiogenesis, and neuroprotection.[77] Neuroglia respond to injury as part of the defence to restore homeostasis; however, when they malfunction, these cells can become a primary pathogenic element.[77]

2.5.2.1 Müller cells

Müller cells are the predominant macroglial element, representing 90% of retinal glia.[77] These cells span from the pigment epithelium to the internal limiting membrane, and their cell bodies sit in the inner nuclear layer. Müller cells have intimate contact with both the retinal blood vessels and the retinal neurons and serve a variety of important functions. Müller cells are involved in; 1) the control of metabolism and supply of nutrients to the retina; 2) the uptake and recycling of neurotransmitters, retinoic acid compounds and ions (such as potassium (K^+)); and 3) the regulation of blood flow and maintenance of the blood-retinal barrier (reviewed by Coughlin et al,[78]).

Müller cells play an essential role in the normal function of a healthy retina. These cells maintain glucose and neurotransmitter homeostasis by removing glucose and the main retinal neurotransmitters glutamate and gamma-aminobutyric acid (GABA) from the extracellular space.[79] Müller cells synthesise, store and degrade glycogen, therefore, when nutrient supplies are low Müller cells utilise glycogen to provide metabolites for other cell types in the retina [80] including photoreceptors which preferentially take up lactate (partially-metabolised glucose produced by Müller cells) as fuel for their oxidative metabolism.[81] Müller cells transform GABA and glutamate into glutamine, which is then returned to the extracellular space and re-utilised by neurons.[81] Additionally, Müller cells induce blood-barrier properties in retinal endothelial cells.[82] While Müller cells play a critical role in maintaining a healthy and functioning retina, abnormalities in any of these functions can result in neuronal dysfunction, cell death, retinal swelling (*i.e.* macular oedema) and break-down of the blood-retinal barrier.[78]

2.5.2.2 Astrocytes

Astrocytes are the second type of macroglial cell and are almost exclusively confined to the innermost retinal layers. A difference between astrocytes and Müller cells is that while Müller cells are distributed throughout the retina independently of vascularisation,[83] astrocytes are present only in vascularised regions.[77] Astrocytes wrap around blood vessels and contact both ganglion and amacrine cells.[77] Like Müller cells, they are key regulators of neuronal nutrition as they store glycogen and provide glucose to neurons. They also serve a role in ionic homeostasis in regulating extracellular K^+ levels and in metabolising neurotransmitters like GABA. Astrocytes also contribute to the integrity of the blood-retinal barrier (reviewed by Wang & Bordey[84]). Of key importance, astrocytes are the main producers of vascular

endothelial growth factor (VEGF) during both normal and pathological vessel formation (reviewed by Penn et al,[85]). Astroglial cells are therefore strongly implicated in vascularisation. While these cells perform essential tasks for the normal physiology of the retina, in response to injury or disease these same astrocytes express several proteins which compromise the integrity of the blood-retinal barrier, upregulate the expression of various genes encoding cytokines, chemokines and elements of the complement cascade, and promote retinal degeneration.[86]

2.5.3 Microglial cells

Microglial cells are found in every layer of the human retina and provide an immunomodulatory function. These cells migrate through the extracellular space between retinal layers and contribute to the healing of degenerating neurons, thereby protecting the functional and structural integrity of the retina.[87] Microglial cells share some of the same properties as circulating and resident macrophages, such as phagocytic activity and the release of various cytokines.[87] Microglial cells are extremely sensitive to changes in their microenvironment and become activated by extracellular signals such as neuronal damage, chronic neurodegeneration, cell death and ischaemia.[87] Once activated, microglia undergo morphological and functional changes, developing into one of two possible phenotypes: an activated pro-inflammatory M1 phenotype which releases inflammatory molecules including cytokines (tumour necrosis alpha (TNF-α), interleukin 1 beta (IL-1β) and interleukin 6 (IL-6)) and toxic metabolites like glutamate. Alternatively, activated microglia can develop an M2 phenotype. These cells are primarily involved in neuroprotection by releasing anti-inflammatory cytokines (IL-4 and IL-10).[88, 89] In summary, microglial activity initially contributes to neuronal protection and tissue regeneration; however, continuous stimulation from exogenous and endogenous stressors can lead to chronic

over activation and loss of these auto-regulatory mechanisms, changes which are detrimental to local neurons.[87, 90] Figure 2.7 is a schematic of the various types of retinal cells, both neuronal and non-neuronal (adapted from Gardner et al,[91]).

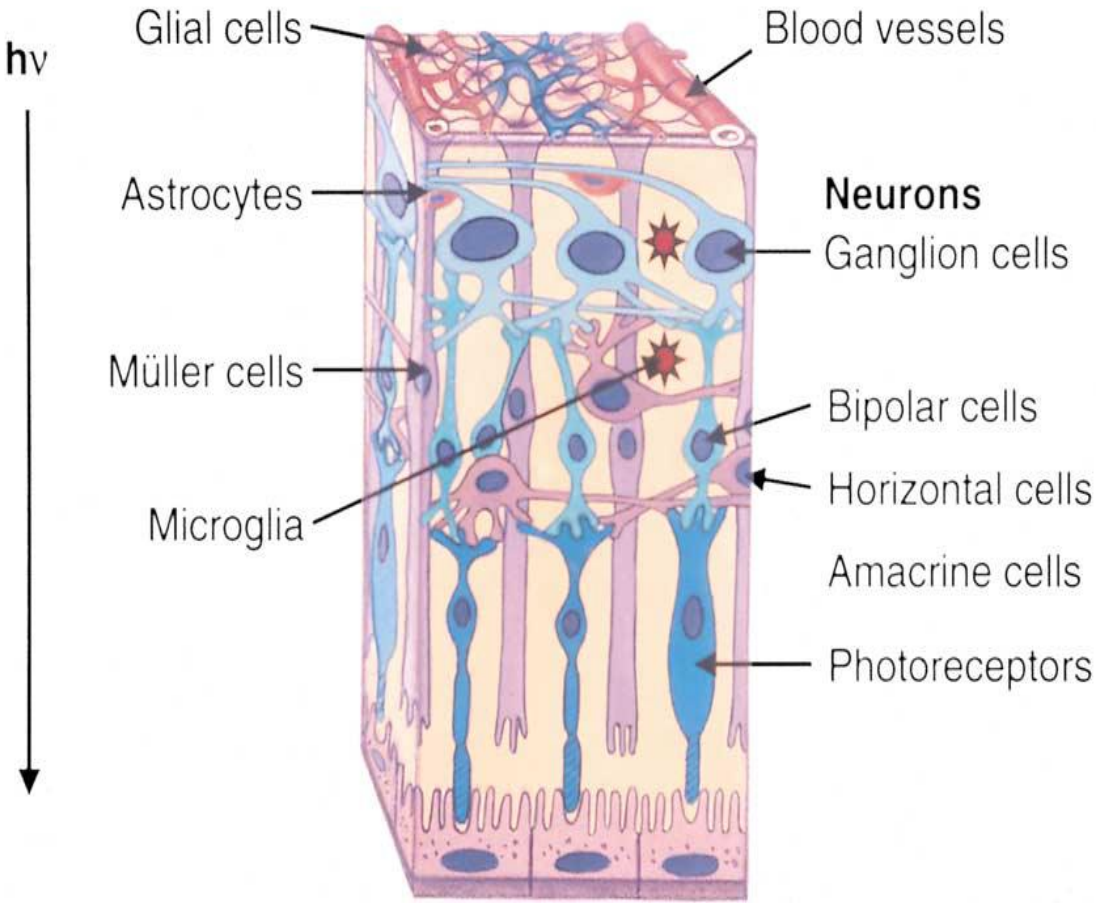


Figure 2.7: Schematic of the major types of retinal cells: vascular; macro glial cells (Müller cells and astrocytes); neurons (photoreceptors, bipolar cells, amacrine, and ganglion cells); and microglia (Adapted from Gardner et al,[91]).

2.5.4 The retinal pigment epithelium

Morphologically the RPE forms a highly polarised epithelial sheet that separates the choroid from the retina. This layer of cells is divided into a basal half which faces the choroid, and an apical half which faces the retina. The basal end of each cell is much

in folded and rests on the basement membrane which forms part of Bruch's membrane. The apical ends of the cells have multiple microvilli and these project between and surround the outer segments of the rods and cones [92] (Figure 2.8). The epithelial cells are joined together by tight junctions which prevent diffusion of large toxic molecules from the choroid capillaries to the photoreceptors; therefore, the RPE acts as a barrier (the outer blood-retinal barrier).[93] The RPE also provides the principal route for the transfer of nutrients to the retina. It takes up nutrients such as glucose, retinol and fatty acids from the blood and delivers these nutrients to the photoreceptors.[94]

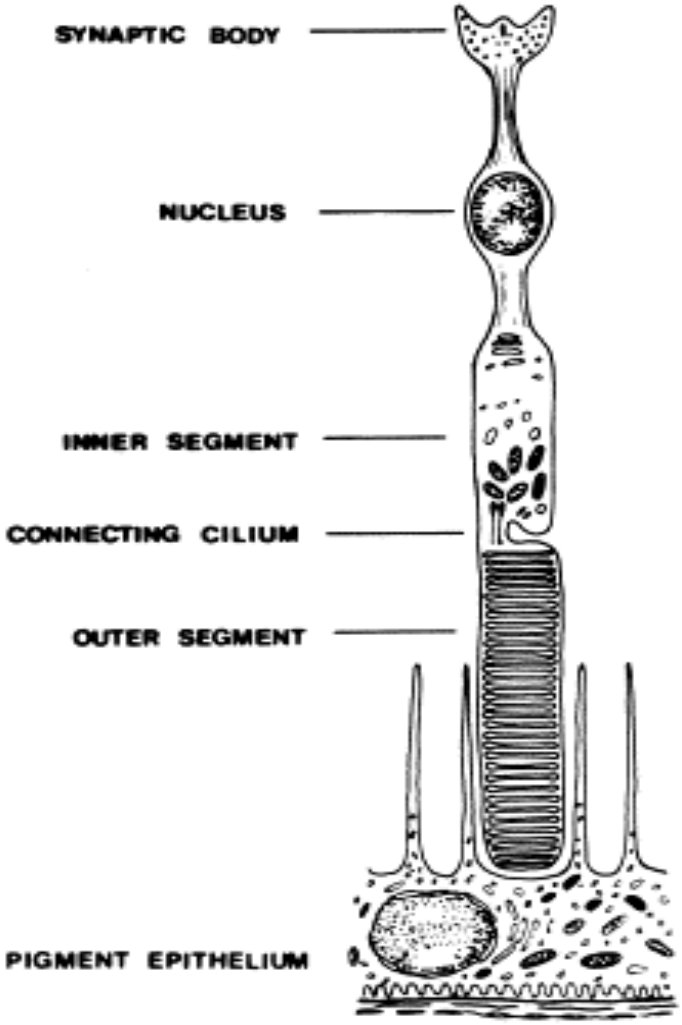


Figure 2.8: Diagram showing the relationship between the photoreceptor cells and the RPE (Adapted from Katz & Robison [95]).

The RPE performs several highly specialised functions essential for homeostasis of the neural retina. The RPE plays an integral part in the visual cycle, as it is the principal storage depot for vitamin A.[93] In this regard, it supplies the essential visual cycle intermediate, 11-cis-retinal, to photoreceptors for the regeneration of rhodopsin.[96] The RPE is also involved in the phagocytosis of shed photoreceptor outer segments.[97] These segments are digested and essential substances, such as retinal, are recycled and returned to the photoreceptors.[98] Additionally, the RPE is known to produce and secrete a variety of growth factors as well as factors which are essential for maintaining the structural integrity of the retina and choriocapillaris, including pigment epithelium-derived factor (PEDF), insulin-like growth factor-1 (IGF-1) and VEGF among others (reviewed by Strauss [93]). The RPE allows oxygen diffusion from the choroidal circulation to the outer retina, a process critical in maintaining normal photoreceptor cell function and survival.[94] Furthermore, as a layer of pigmented cells, the RPE protects the retina from light damage by the absorption of excessive photons.[99] The many complex and unique functions of the RPE play a critical role in vision. A failure of any one of these functions can lead to degeneration and loss of visual function; however, to date, the role of the RPE in diabetic retinopathy remains elusive.

2.5.5 Endothelial cells and pericytes

The fifth class of cells includes vascular endothelial cells and pericytes. Retinal capillaries are comprised of a single layer of endothelial cells bound by pericytes, and both cell types are covered by a common basement membrane[100] (Figure 2.9). Interaction between pericytes and vascular endothelial cells is important in the regulation of vascular function and the maintenance of the retinal homeostatic environment. Endothelial cells line the vasculature and serve as a physical barrier

between blood and the surrounding tissue and constitute the inner blood-retinal barrier.[91] The presence of tight junctions prevents the outward flow of macromolecules and maintains the local microenvironment.[101] The endothelium also efficiently supplies oxygen and nutrients to the neural retina. Under certain conditions such as hyperglycaemia, however, retinal endothelial cells become particularly vulnerable.

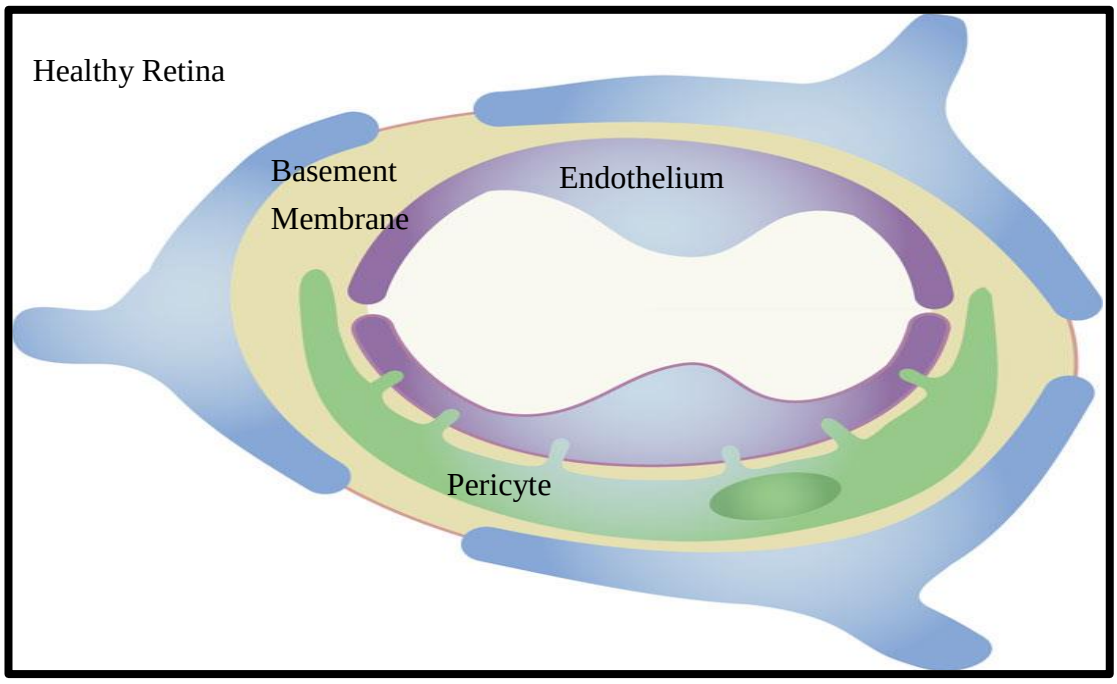


Figure 2.9: Endothelial–pericyte interactions in micro vessels. Pericytes surrounding endothelial cells share the basement membrane with endothelial cells (Modified from Simo et al,[100]).

Pericytes are modified smooth muscle cells that line the outer walls of capillaries and play a critical role in vessel patency by regulating vascular tone and providing structural support.[102] In this way, pericytes regulate retinal vascular flow by dilating and contracting,[91] and provide nutritional support and waste product removal for the inner retina.[29] One of the earliest responses to hyperglycaemia is pericyte apoptosis.[103]

Endothelial cells' and pericytes' wide-ranging functions include mediating repair of the vasculature, promoting the blood-retinal barrier and functioning as hypoxic sensors.[104]

Retinal microvascular dysfunction is a major component of diabetic retinopathy and is characterised clinically by dot and blot haemorrhages and micro aneurysms on the retina in the earlier stages of diabetes. Visible retinal changes have led to the general assumption that diabetic retinopathy is predominantly a microvascular abnormality, however, it is now evident that diabetic retinopathy affects all of the major retinal cell types (including neuronal and non-neuronal cells). Both retinal neurodegeneration and microvascular complications are therefore implicated in diabetic retinopathy.[91] The interaction and functional integration of all of these cell types is required for normal vision, however, disruption to any of them may impair vision.

2.5.5.1 The blood-retinal barrier

Metabolic support for the inner retina comes from a vascular network that traverses the ganglion cell layer and extends down to the outer plexiform layer. Support for the outer retina is achieved by diffusion from the vascularised choroid across the RPE. Together, these retinal vessels along with the RPE form the inner and outer blood-retinal barrier.[105, 106] The blood-retinal barrier partitions the neural elements of the retina from the circulation to protect it from circulating inflammatory cells and their cytotoxic products, and allows the retina to regulate its extracellular composition.[105] This functional barrier resides at the level of tight junctions between adjacent endothelial cells.[91] Several unique proteins constitute the vascular endothelial tight junctions including occludin and claudins.[107] These proteins span the plasma membrane and limit fluid flow between endothelial cells [106] (Figure 2.10).

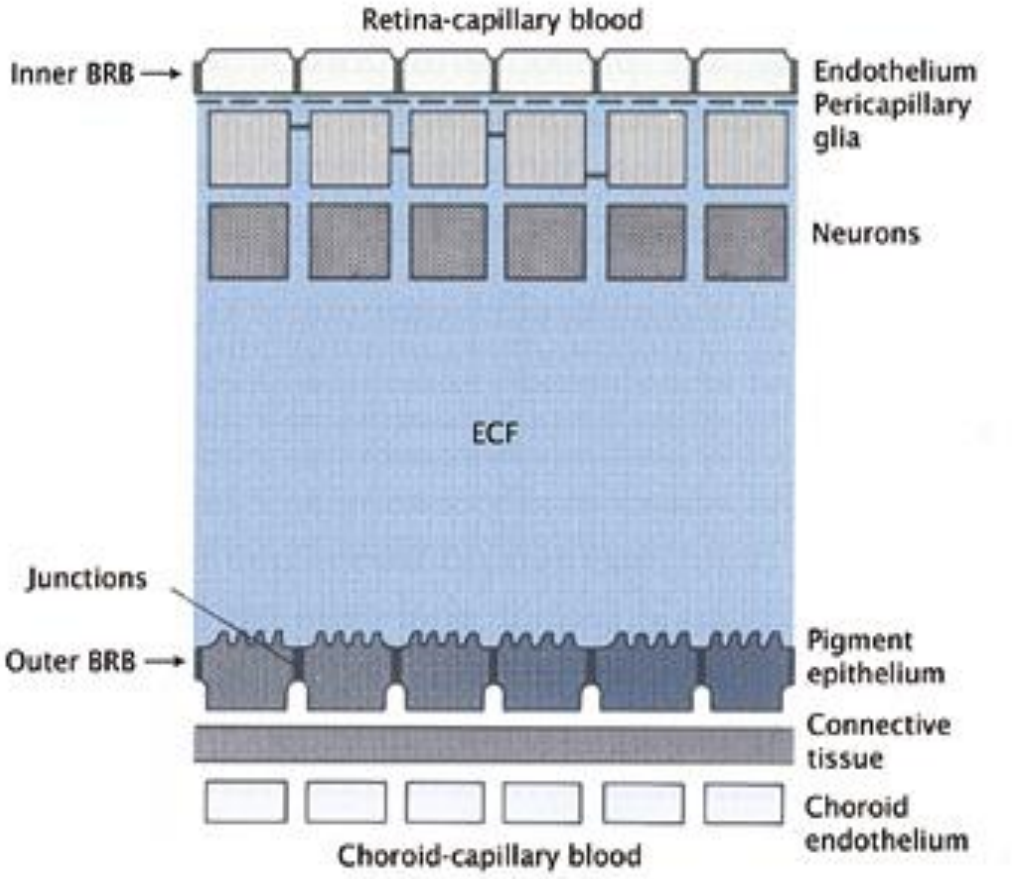


Figure 2.10: Schematic presentation of the inner and outer blood-retinal barriers and their relative location (Adapted from Cuhna-Vaz et al, [106]). Abbreviation: ECF = extracellular fluid.

The cytokine, VEGF, can induce changes in the expression of these proteins and therefore influence vascular permeability.[105] Because all retinal cells (macroglial, microglial, and neurons) are capable of releasing pro-inflammatory cytokines such as VEGF, it is likely that they further exacerbate retinal vascular permeability in diabetes.[101]

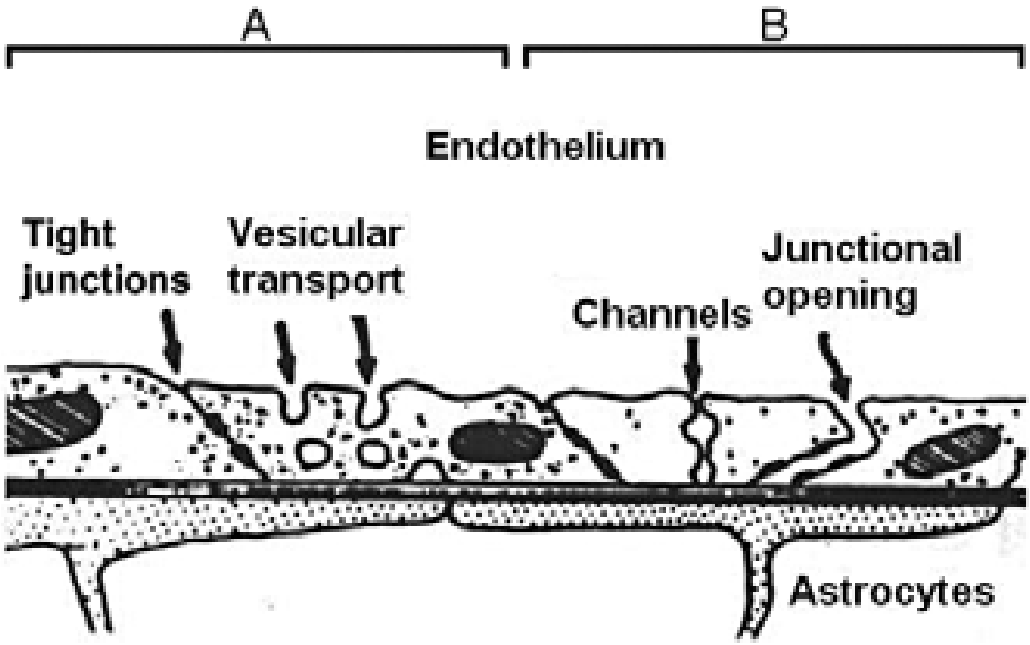


Figure 2.11: Pathways for solute movements across the inner blood-retinal barrier (retinal endothelial cells). (A) Normal. (B) Mechanisms of the breakdown of the inner blood-retinal barrier (Adapted from Cunha-Vaz et al,[106]).

2.6 Blood supply to the retina

The retina receives vascular supply from two sources: the central retinal artery supplies the inner retina and the choriocapillaris supplies the RPE and outer retina (both of which originate from the ophthalmic artery). The retina is one of the most metabolically active tissues in the body, therefore, the retina must have an extensive vascular network to maintain proper functioning and to cope with such a high oxygen demand. The central retinal artery enters the optic nerve some 10 to 15 mm behind the globe. On entering the eye, the vessels branch into inferior and superior divisions which continue to subdivide into arterioles (superior, inferior, nasal and temporal), and extend away from the optic disc to supply the inner two-thirds of the retina [31] (Figure 2.12).

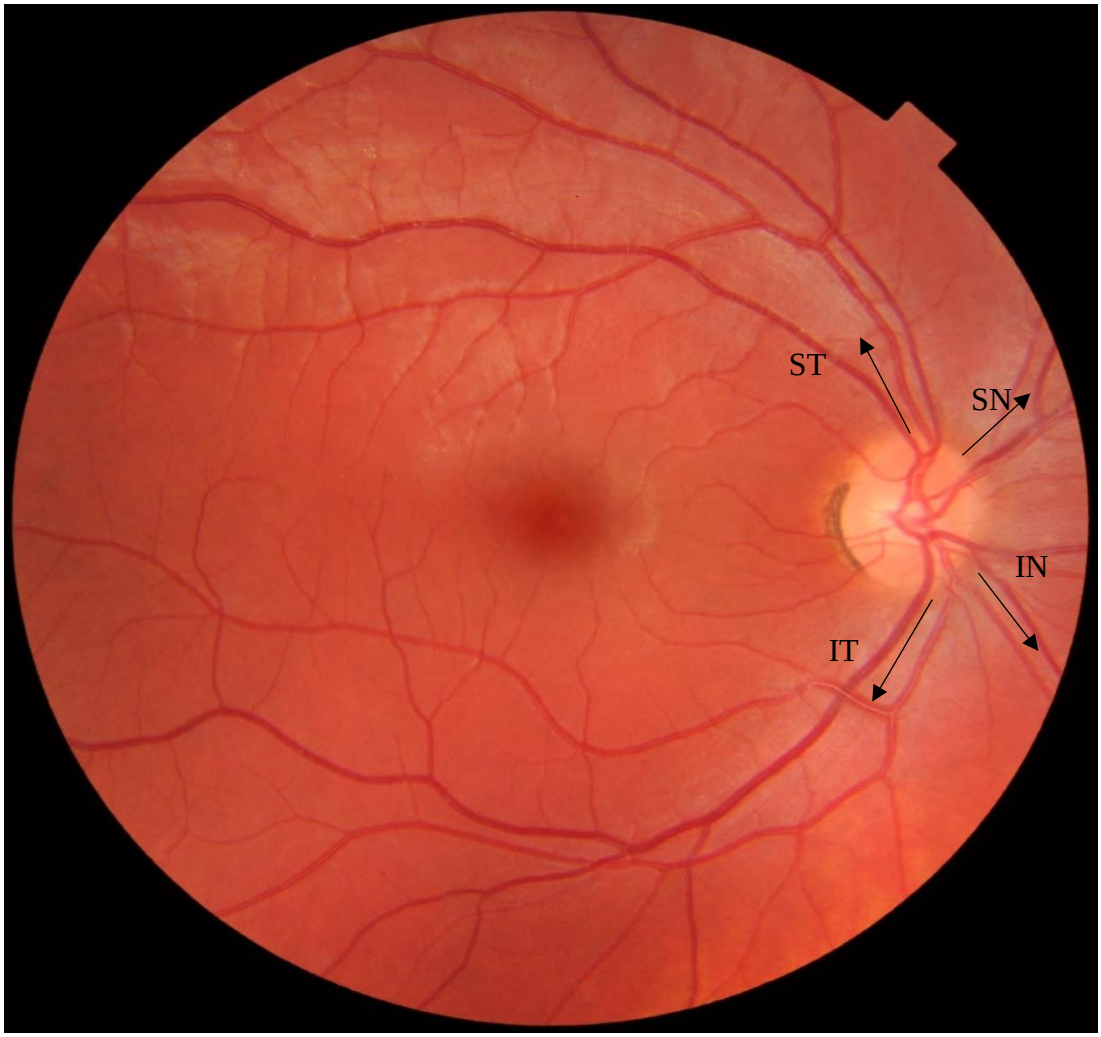


Figure 2.12: A fundus photograph showing the retinal blood supply to the superior temporal (ST), superior nasal (SN), inferior temporal (IT) and inferior nasal (IN) arcades.

The arterial intra-retinal branches supply three layers of capillary networks: the radial peri-papillary capillaries and the inner and outer layer of capillaries [108] (Figure 2.13). The retinal vascular vessels provide blood to the inner retinal neurons which receive 20-30% of the total ocular blood flow.[31]

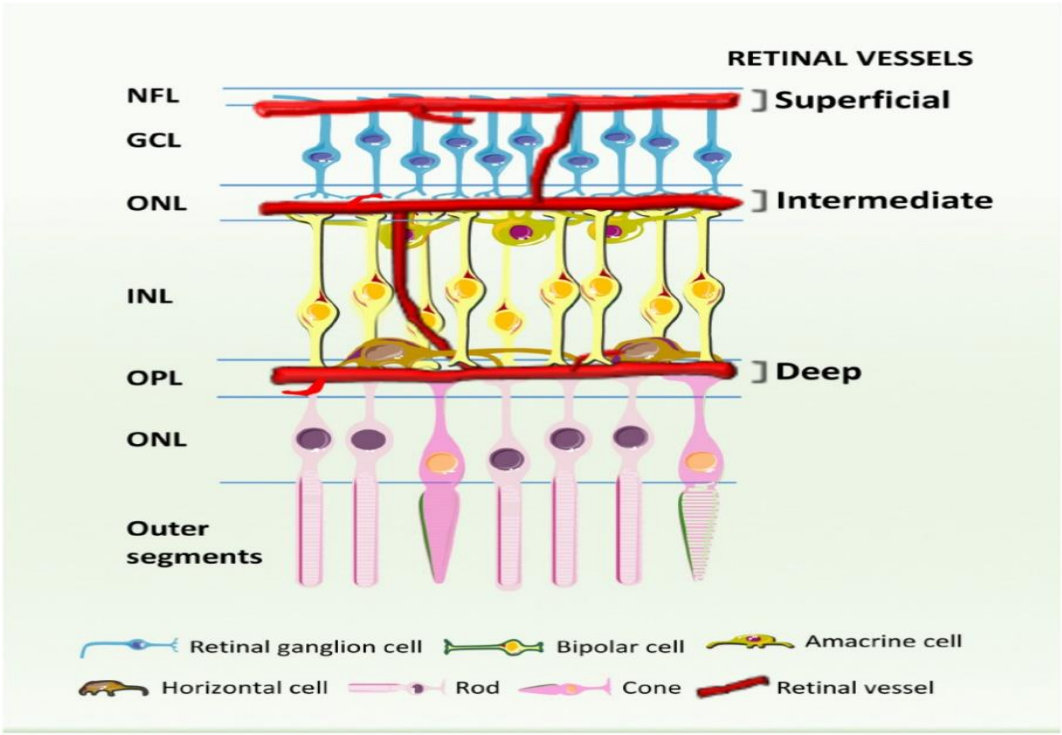


Figure 2.13: Schematic cross-section showing the retinal blood vessels lining the inner surface of the retina (Adapted from Santiago et al, [101]).

Three capillary plexuses are embedded among retinal neurons: the superficial layer lies within the inner nerve fibre layer (NFL) with branches extending into the ganglion cell layer (GCL), while the intermediate and deep capillary plexuses align along each side of the inner nuclear layer (INL). ONL, outer nuclear layer; IPL, inner plexiform layer; OPL, outer plexiform layer.

The choroidal network supplies the choroid and the outer third of the retina with oxygen and other necessary nutrients. The choroid receives 65-85% of ocular blood flow,[31] and is vital for the maintenance of the avascular outer retinal layers, particularly the photoreceptors. The choroid is supplied primarily by the long and short posterior ciliary arteries (branches of the ophthalmic artery), with a minor contribution

from the anterior ciliary arteries.[31] The choriocapillaris supplies the RPE and the outer retinal layers. Retinal vessels are absent centrally at the fovea, (*i.e.* the FAZ) and the choroid is responsible for blood supply to this region (Figure 2.14). The foveola has the highest perfusion rate of any vascular bed within the human body reflecting the high metabolic activity of the photoreceptors.[109]

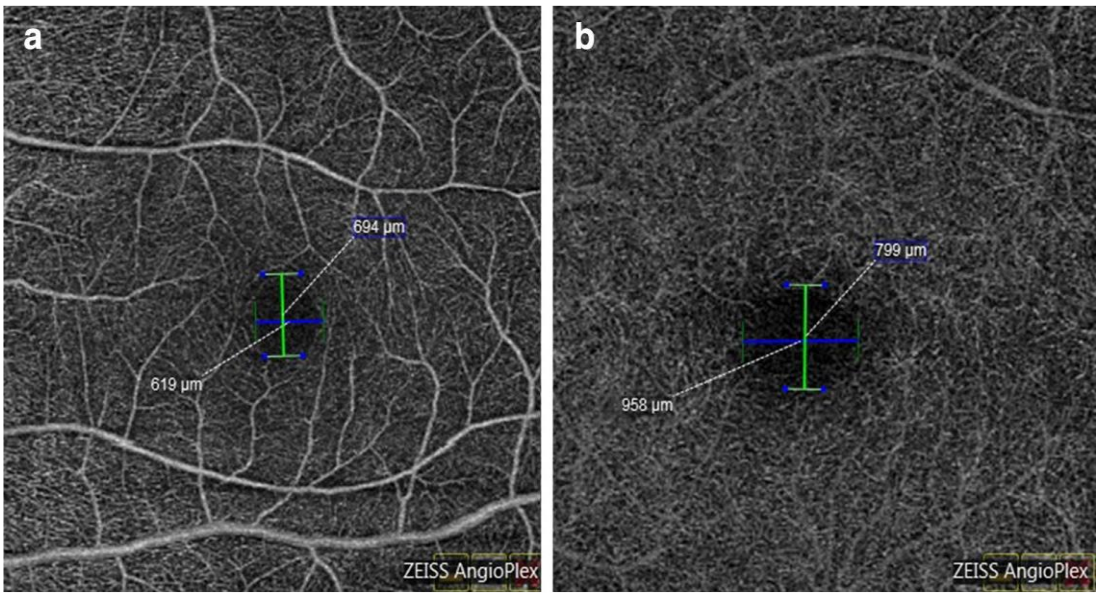


Figure 2.14: Image of the FAZ. The calliper measurement of both horizontal and vertical diameters of superficial (a) and deep (b) capillary plexus of the FAZ (Adapted from Hussain & Hussain [110]).

The retinal venous system exits the eye via the central retinal vein. The corresponding retinal venous branches have much the same distribution as the retinal arteries. Choroidal blood is thought to be drained exclusively through the vortex veins, with one or two vortex veins draining each of the four quadrants of the eye.[111] The vortex veins penetrate the sclera to merge with the ophthalmic vein.

2.6.1 Retinal oxygen consumption

Retinal oxygen distribution varies across the retina according to retinal cell type and their associated level of metabolic activity. The highest oxygen tension is found in the choroid and the lowest at the level of the photoreceptor inner segments.[112] While the choroid has a high oxygen tension, the extraordinarily high oxygen demand of the photoreceptors, combined with the avascular nature of the foveola, results in a substantially increased risk of these cells experiencing hypoxic episodes.[112]

2.7 Vulnerability of the retina to diabetic retinopathy

The physiology of the retina, and the unique structure of the macula, in particular, may contribute to the eye's susceptibility and vulnerability to diabetic retinopathy.[29] The central retina is adapted for high acuity vision. To maximise vision, there is a requirement for transparency at the macula, and this transparency imparts several anatomical and physiological constraints in this region including; 1) the central retina is devoid of blood vessels (*i.e.* the FAZ), an anatomic specialisation that serves to enhance visual acuity (VA) by removing angioscotomas; [42] 2) the retinal axons are not ensheathed by myelin, as myelin is opaque and would, therefore, block light transmission; [29] and 3) there is a reduced number of mitochondria in the inner retina, as mitochondria contain light-absorbing haem-based cytochrome proteins which would impede vision.[29] These structural arrangements and adaptations are necessary to ensure undisturbed light transmission at the point of highest VA, at the foveola.

Retinal and neural adaptations, such as these may, however, increase the eye's vulnerability to oxidative stress and inflammation.[29] The partial oxygen pressure declines from the outer retina to the inner retina.[113] The oxygen tension of the inner retina is relatively hypoxic because of the reduced density of blood vessels at the

foveola. Furthermore, the inner retina possesses relatively few mitochondria. Consequently, the inner retina relies heavily on anaerobic respiration (*i.e.* glycolysis) which is a less efficient means of generating energy, compared with aerobic respiration (*i.e.* oxidative phosphorylation) which predominates in the outer retina.[29] Furthermore, demyelinated retinal nerves require more energy to maintain membrane potentials compared with myelinated axons.[114]

Despite the sparse vascularity and low oxygen tension, the retina has one of the highest metabolic demands of any tissue in the body.[112] Energy is required for phototransduction, for neurotransmission at synapses, to replenish photoreceptor outer segment membranes (daily), and to transport proteins and neurotransmitters via axons to the optic nerve and lateral geniculate body of the thalamus.[29] A healthy, visually-functioning retina requires intact cell-cell communication, a specialisation that may, however, be vulnerable to the hyperglycaemia-induced metabolic perturbations typical of diabetes, which may result in neurotoxic or pro-inflammatory responses. Interestingly, the outer retina is relatively protected from the early insults of diabetes as it receives its oxygen and nutrients by diffusion from the choroid through the pigmented epithelium. The combination, however, of high metabolic demand of the retinal neurons, low vascular supply, and the presence of few mitochondria may limit the inner retina's ability to adapt to chronic hyperglycaemia in diabetes and its associated oxidative stress/inflammation in the pathogenesis of diabetic retinopathy.

3. DIABETES MELLITUS

3.1 Introduction to diabetes mellitus

Diabetes mellitus (diabetes) is a complex metabolic disorder, characterised by the elevation and dysregulation of blood glucose levels as a result of a lack or insufficiency of insulin.[24] The condition is broadly classified into Type 1 and Type 2 diabetes.[27] Type 1 diabetes is less common accounting for approximately 10 % of all diabetes cases. It results from the autoimmune destruction of pancreatic beta (β)-cells,[115] leading to complete dependence on exogenous insulin to regulate blood glucose levels.[116] Type 1 diabetes typically presents in childhood or young adulthood. Type 2 diabetes mellitus is the more common form of the disease accounting for approximately 90% of patients.[117] It normally affects individuals in later life, however, due to increasing prosperity, lifestyle changes, and obesity, Type 2 diabetes is now affecting a much younger age group.[118] Type 2 diabetes is characterised by insufficient secretion of insulin from the β-cells of the pancreatic islets, coupled with impaired insulin sensitivity in target tissues such as muscle, liver and adipose tissue (a condition termed insulin resistance).[119] Therapy for Type 2 diabetes consists initially of dietary control and lifestyle modification, followed by oral hypoglycaemic agents, which may reduce insulin resistance, increase insulin secretion or reduce hepatic glucose output.[120] If these treatments fail to control hyperglycaemia, then insulin is given. Both forms of diabetes (Type 1 and Type 2) are caused by a combination of genetic and environmental risk factors.

The pathogenesis of Type 1 and Type 2 diabetes is different. Type 1 diabetes is a chronic disease that destroys the body's ability to make insulin. Young patients with suspect Type 1 diabetes require prompt referral and diagnosis or else they risk diabetic

ketoacidosis, a potentially life-threatening condition. Diabetic ketoacidosis can develop when the body fails to store blood glucose as glycogen in the fed state due to a lack of insulin; in this scenario, the body metabolises fat in a non-physiological manner producing ketones to fuel the brain and other tissues which normally use glucose. Unabated, this adaptive process results in a build-up of ketones in the body with toxic effects,[121] giving rise to diabetic coma or even death in extreme circumstances. Once diagnosed, patients with Type 1 diabetes require a lifetime of treatment with insulin.[122]

Type 2 diabetes usually develops insidiously in adulthood, although it is now affecting a much younger age group. In Type 2 diabetes the pancreas usually produces some insulin, however, the amount produced is not enough to overcome the resistance of target tissues to its effects. In an insulin resistance state, normal or elevated insulin levels consequently produce an attenuated biological response.[123] The relative timing of β-cell dysfunction and insulin resistance can, however, vary and it has been shown that 'abnormal' insulin sensitivity can precede the clinical diagnosis of diabetes by up to 15 years.[124] The failure of compensatory hyperinsulinaemia by the pancreas is the 'hallmark' of frank hyperglycaemia [125] and Type 2 diabetes ensues. At the time of diagnosis patients with Type 2 diabetes generally present with a series of metabolic comorbidities, including, insulin resistance, hyperinsulinaemia, hyperlipidaemia, hypertension and overweight/obesity, features which are less common in Type 1 diabetes at diagnosis.[126] Abnormal glucose metabolism can exist for many years in Type 2 before overt diabetes is diagnosed and there may be no initial symptoms.[24] Lifestyle modification early on, however, may not only address the existing hyperglycaemia and other metabolic abnormalities but may also lead to better visual

outcomes.

3.2 Epidemiology

With life expectancy and obesity on the rise worldwide, diabetes mellitus is becoming a major public health problem, approaching epidemic proportions. Globally, an estimated 422 million adults were living with diabetes in 2014, compared to 108 million in 1980.[127] The prevalence (age-standardised) of diabetes worldwide has nearly doubled since 1980, rising from 4.7% to 8.5% in the adult population.[128] The number of patients with diabetes is predicted to rise to 642 million (uncertainty interval: 521–829 million) by 2040.[127] The International Diabetes Federation Diabetes Atlas estimated that 207,490 people in the 20 – 79 year age group were living with diabetes in Ireland in 2013 (prevalence 6.5%).[129] A study carried out by TILDA in 2015, estimated that the prevalence of Type 2 diabetes and pre-diabetes was 9.5% and 5.5% respectively, with approximately 10% of cases undiagnosed.[130] According to the Healthy Ireland survey, 854,165 adults over 40 in the Republic of Ireland have Type 2 diabetes or are at increased risk of developing it.[131] Furthermore, there are 304,383 people in the 30-39 year age group who are overweight and not taking the recommended quantum of weekly physical activity, leaving them at an increased risk of developing Type 2 diabetes.[131] Therefore, there are over a million adults in Ireland who need to make changes to their habitual dietary and physical activity patterns in order to address their elevated risk of diabetes.[131]

Diabetic retinopathy, a common and specific microvascular complication of diabetes, remains the leading cause of blindness in working-aged people.[25] Up to 21% of patients with Type 2 diabetes have retinopathy at the time of diagnosis.[25] After 20 years with diabetes, nearly all patients with Type 1 and more than 60% of Type 2

patients have developed retinopathy,[25] and this prevalence increases with advancing age.[132] Early detection of retinopathy in individuals with diabetes is critical in preventing vision loss.[133] Irish statistics show that on average one person with diabetes goes blind in Ireland each week.[134] The National Diabetic Retinal Screening Programme was developed in Ireland in 2013 and offers free regular diabetic retinopathy screening to all people with diabetes aged 12 and over. Optometrists play an important role in ensuring that patients participate. While there is no cure for diabetic retinopathy, prompt treatment may be effective in preventing, delaying or reducing vision loss in patients with this diabetes complication.

3.3 Diabetic retinopathy

Diabetic retinopathy is a major complication of diabetes mellitus and remains a leading cause of preventable blindness.[135] Traditionally it has been viewed as a microvascular disease where microvascular lesions have been used as the major criterion for evaluating and classifying diabetic retinopathy. Clinically, diabetic retinopathy can be divided into two stages: non-proliferative and proliferative diabetic retinopathy.[136] Non-proliferative retinopathy is characterised by damage to the retinal vasculature, increased vascular permeability, thickening of the basement membrane and loss of pericytes.[137] It can be classified into mild (micro-aneurysms), moderate (micro-aneurysms, retinal haemorrhages or hard exudates) and severe (20 haemorrhages in each of the four quadrants, venous beading in two quadrants or the presence of intra-retinal microvascular anomalies) [136] (Figure 3.1).

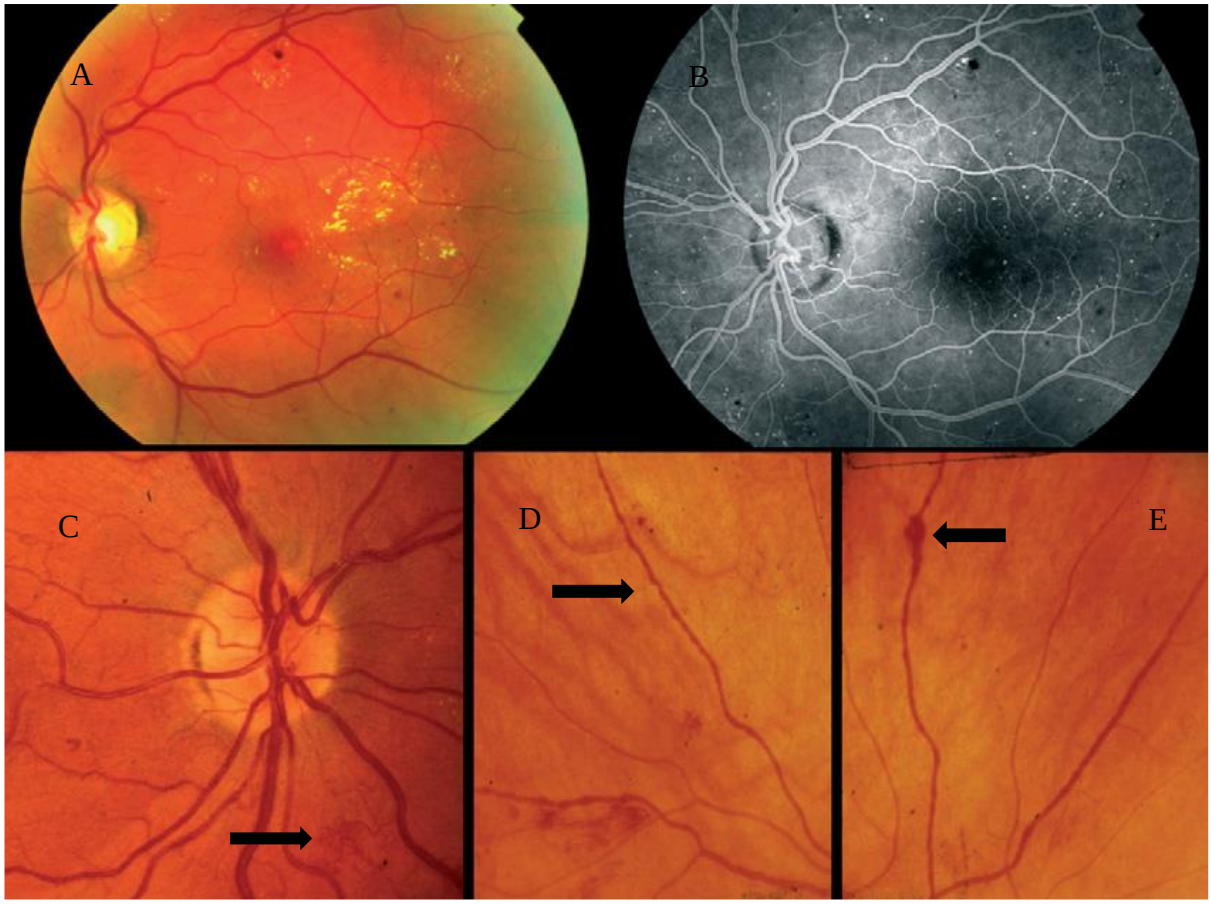


Figure 3.1: Non-proliferative diabetic retinopathy; retinal micro-aneurysms, haemorrhages and hard exudates (A and B); intra-retinal microvascular abnormalities (C arrow); venous beading (D arrow) and venous loop formation (E arrow) (Modified from Cheung et al,[138]).

Proliferative diabetic retinopathy is marked by the growth of new vessels on the retina and posterior surface of the vitreous (angiogenesis), vitreous haemorrhage, retinal scars and detachment.[136] The ischaemic retina contains high levels of VEGF, the driving force for neovascularisation.[139] This stage of the disease can result in profound and irreversible vision loss (Figure 3.2).

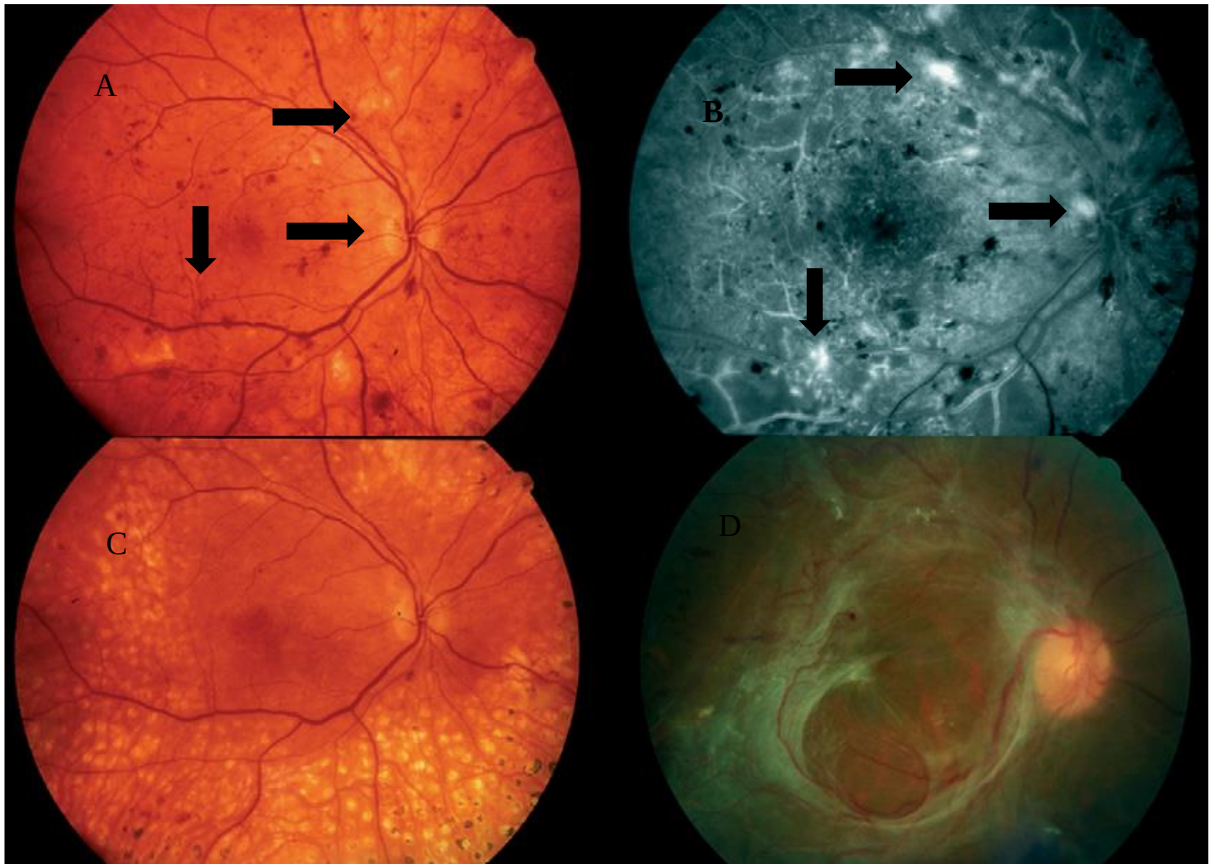


Figure 3.2: Proliferative diabetic retinopathy; neovascularisation is a hallmark of proliferative diabetic retinopathy (A, arrows). It can also be identified on fluorescein retinal angiogram (B, arrows). Resolution of retinopathy with pan-retinal photocoagulation (C). Progression of retinopathy without treatment (D) (Modified from Cheung et al,[138]).

Diabetic macular oedema, a side effect of both non-proliferative and proliferative diabetic retinopathy, can occur at any stage of the disease.[137] It is caused by increased vascular permeability and leakage of proteins and lipids into the extravascular space due to functional damage of the retinal vascular epithelium and a breakdown of the blood-retinal barrier [137] (Figure 3.3).

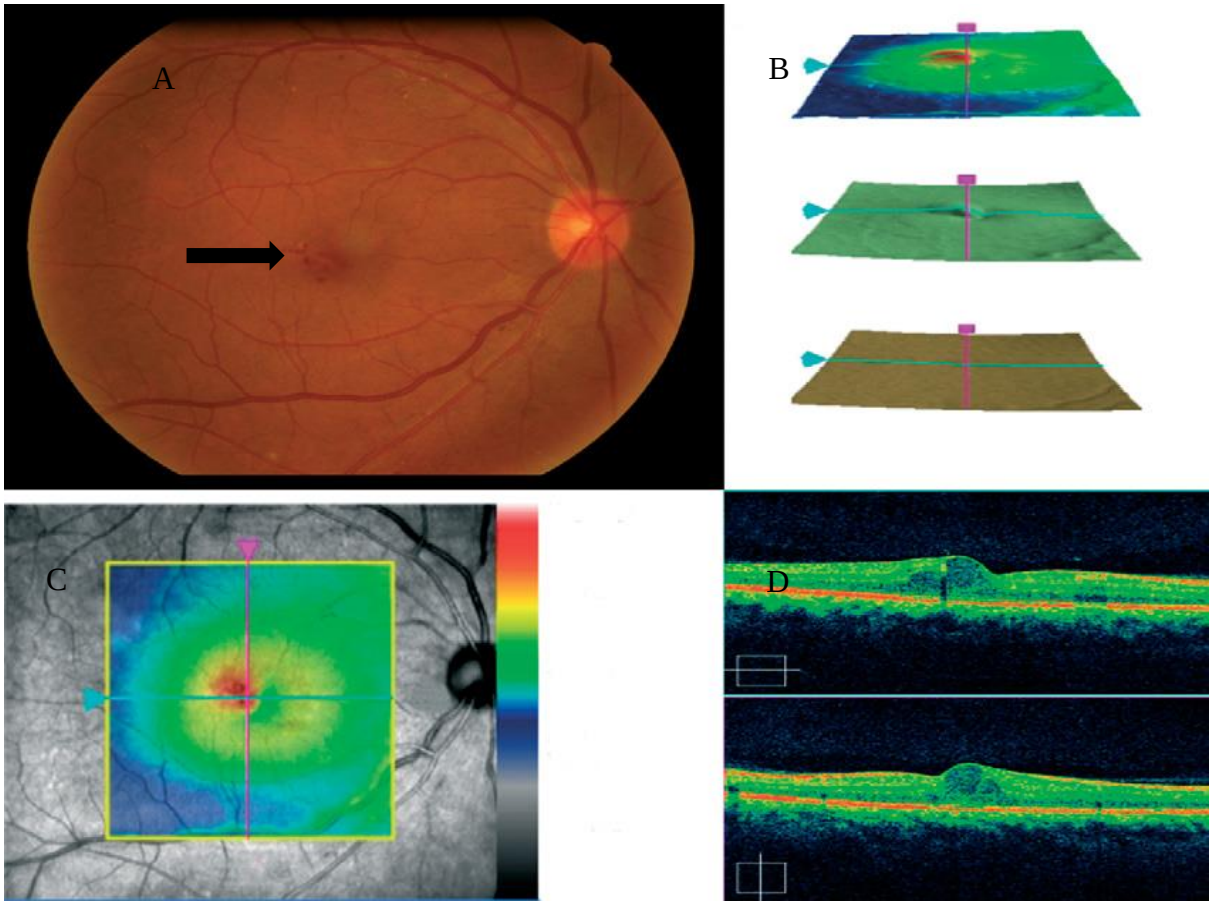


Figure 3.3: Diabetic macular oedema; clinical diagnosis of diabetic macular oedema based on stereoscopic examination of the macula (A, arrow). Diagnosis can also be supplemented by OCT; three-dimensional topographic maps of the macula from OCT allow visualisation of macular oedema in relation to internal limiting membrane or RPE; B; overall topographic image (C), and horizontal (upper image, D) and vertical (lower image, D). Cross-sectional images of the macula from OCT allow quantitative and qualitative assessment of macular oedema (Modified from Cheung et al,[138]).

Improving technology has enabled the detection of more subtle pathologies and neural layer abnormalities in patients with diabetes. Optical coherence tomography angiography (OCTA) is a new vascular imaging technique which allows practitioners to non-invasively investigate the FAZ and perifovea in more detail. Non-perfusion

indices and FAZ parameters have been found to correlate with glycated haemoglobin (HbA1c) (a measure of the patient's average glucose level over the previous 2 to 3 months), in those with non-proliferative diabetic retinopathy.[140] Early detection of diabetic retinopathy can prevent severe loss of vision and even blindness.

3.4 Pathophysiology of diabetic retinopathy

Diabetic retinopathy has long been recognised as a microvascular disease. Chronic exposure to hyperglycaemia and other causal factors (e.g. hypertension, dyslipidaemia) are believed to initiate a cascade of biochemical and pathophysiological events which ultimately lead to microvascular changes and retinal dysfunction. An understanding of the pathological processes leading to the onset of diabetic retinopathy may provide the means to better manage and treat the disease. A summary of these processes are presented here.

3.4.1 Hyperglycaemia

The initial stages of retinopathy collectively relate to endothelial dysfunction (*i.e.* leukocyte adhesion, blood flow alterations, capillary closure and formation of leaky vessels) and are most probably caused by the effects of elevated blood glucose concentration on microvascular tissues.[141] Adverse effects of persistently elevated glucose levels vary according to cell type. Cells expressing high levels of the glucose transporter 1 (GLUT 1), such as vascular endothelial cells, are unable to regulate intracellular glucose concentrations and are, therefore, more susceptible to hyperglycaemia-induced damage.[142] The presence of hyperglycaemia affects various molecular pathways, ultimately resulting in a breakdown of the blood-retinal barrier, and damage to cells that make up these structures, including endothelial cells and pericytes.[143] Multiple metabolic pathways have been implicated in hyperglycaemia-

induced microvascular damage and these include the polyol pathway, activation of advanced glycation end products (AGEs), and the hexosamine and protein kinase C (PKC) pathways (Figure 3.4).[144]

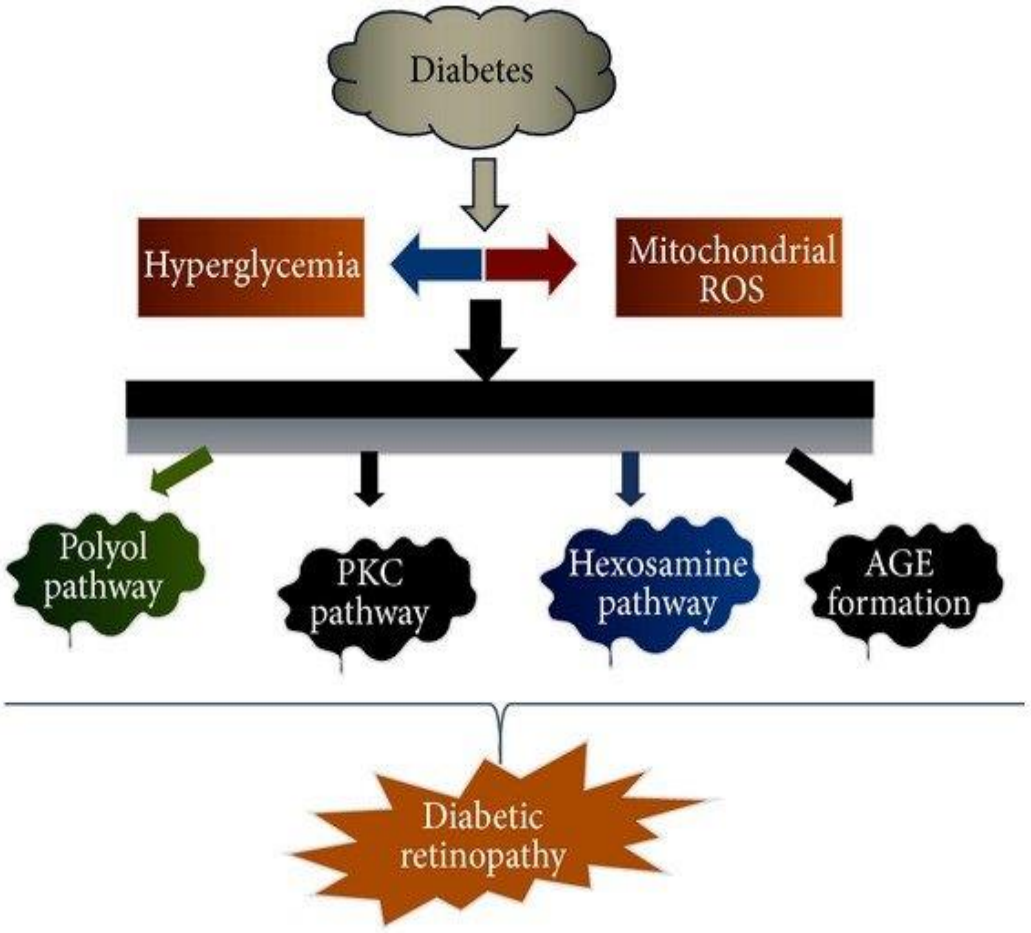


Figure 3.4: The four major mechanisms involved in diabetic retinopathy are increased polyol pathway flux, increased AGE formation, activation of PKC, and hexosamine pathways (Adapted from Safi et al,[144]).

These pathways in one way or another result in increased oxidative stress, inflammation and vascular occlusion, factors which contribute to the pathogenesis of diabetic retinopathy. Several significant studies have provided strong evidence that tight control of blood glucose, as assessed by HbA1c, can delay diabetic retinopathy

onset and progression in patients with Type 1 [28] and Type 2 [145] diabetes. The Diabetes Control and Complications Trial (DCCT) showed that intensive glycaemic control reduced the incidence of retinopathy by 76%, and progression from early to advanced retinopathy by 54% [28] highlighting that strict glycaemic control is much more effective in preventing and delaying the onset of diabetic retinopathy in patients with diabetes without retinopathy, compared with limiting the severity of retinopathy once it occurs.

Notwithstanding these findings, research has also shown that if blood glucose is rapidly controlled in patients with previously poor control, it can worsen diabetic retinopathy.[146] Furthermore, there are a group of diabetes patients who are unable to prevent the onset or progression of diabetic retinopathy despite achieving good metabolic control of the disease.[146] These findings highlight that hyperglycaemia does not fully explain the wide range of functional and cellular changes which appear over the course of diabetic retinopathy, and that other pathogenic components of diabetes may also contribute to its progression.

3.4.2 Hypertension

Hypertension and diabetes are common, intertwined, conditions that share a significant overlap in their underlying risk factors (including familial predisposition, advancing age, dyslipidaemia and obesity, among others); and in their complications (microvascular and macrovascular disorders).[147, 148] Up to 75% of adults with diabetes also have hypertension, and patients with hypertension alone often show evidence of insulin resistance.[147] Moreover, as hypertension and diabetes are both components of the metabolic syndrome, these conditions commonly co-occur.[147, 149]

Oxidative stress plays an important pathophysiological role in hypertension. Normally

reactive oxygen species (ROS) are produced in the vessel wall in a controlled and tightly regulated manner; however, under pathological conditions (*i.e.* hypertension), increased production of ROS leads to endothelial dysfunction, impaired vascular relaxation, increased smooth muscle growth, and hypertrophy.[150] Blood flow and blood pressure can cause internal stresses in vessels (such as endothelial shear stress and circumferential wall stress respectively).[151] Figure 3.5 shows the forces created by flowing blood.

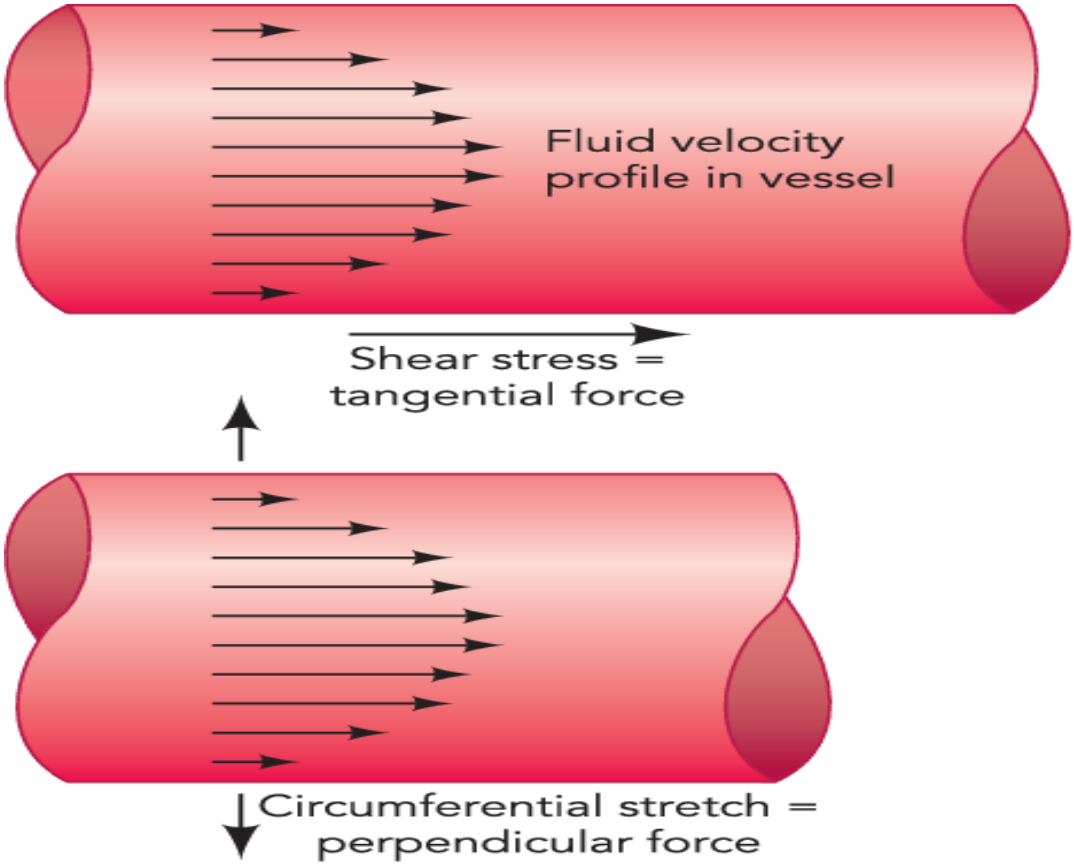


Figure 3.5: Fluid flow within a tube creates two types of forces: shear stress, which is a force tangential to the vessel wall, and pressure, which creates a circumferential stretch perpendicular to the vessel wall (Adapted from Jones et al,[152]).

Hypertension exacerbates diabetic retinopathy through increased blood flow (shear stress) and pressure (circumferential wall stretch) of vascular endothelial cells.[151,153, 154] Collectively, oxidative balance regulates the degree of tone or vasodilation, and this balance is upset in hypertension. Treatment with antioxidants or agents to inhibit nicotinamide adenine dinucleotide phosphate (NADPH) oxidase has been shown to decrease ROS production, prevent target-organ cellular damage and decrease blood pressure in animal models and human hypertension (reviewed by Touyz & Shiffrin [155]). Effective treatment of hypertension (goal blood pressure less than 140/85 mmHg) has been shown to reduce the rate of worsening of diabetic retinopathy by 34 % over 9 years follow-up,[156] and to reduce the risk of vision loss by three lines or more by 47%.[156] The recommended guidelines, according to the American Diabetes Association, for systolic blood pressure and diastolic blood pressure levels are <130 mmHg and <85 mmHg respectively.[157] The role of hypertension in diabetes will be explored in more detail in chapter seven.

3.4.3 Dyslipidaemia

Hyperglycaemia and defects in insulin action may also lead to changes in plasma lipoproteins in patients with diabetes.[158] Additionally, the obesity/insulin-resistant metabolic disarray commonly associated with Type 2 diabetes, may itself, lead to lipid abnormalities exclusive of hyperglycaemia.[158, 159] Dyslipidaemia is characterised by a moderate elevation in TGs, low HDL cholesterol and the presence of small, dense, atherogenic LDL particles,[160] (reviewed in chapter six). In brief, dyslipidaemia often arises in untreated or uncontrolled diabetes, or in people who become overweight and develop insulin resistance.[161] Impairment of the biological action of insulin at a cellular level (insulin resistance) is thought to primarily underlie the metabolic defects which contribute to the development of dyslipidaemia.[161] Insulin plays a central role

in how the body utilises and stores glucose and fat. Insulin suppresses lipolysis in adipocytes via its effect on hormone-sensitive lipase (HSL), and it also stimulates the action of lipoprotein lipase (LpL), an important enzyme in the hydrolysis of plasma TGs and free fatty acid (FFA) delivery to adipocytes.[158] In an insulin-resistant state, (*i.e.* Type 2 diabetes, overweight/obesity), there is an increase in intracellular hydrolysis of TGs and the release of FFAs into circulation from adipose tissue. Furthermore, insulin activation of LpL is delayed.[162] Overall, this culminates in an increased concentration of FFAs in circulation returning to the liver, where they are reassembled into TGs. The presence of excess TGs in the liver promotes TG-rich very-low-density lipoprotein (VLDL) production,[163, 164] and the accompanying hepatic insulin resistance fails to effectively suppress the release of TG rich VLDL in circulation.[165]

Heterogeneity exists in the size and composition of all classes of lipoproteins. The core of lipoproteins contain hydrophobic cholesterol ester and TGs, and their proportions are determined by the enzyme cholesteryl ester transfer protein (CETP). In the presence of increased concentrations of VLDL in circulation, CETP will exchange VLDL TGs for cholesteryl ester in the core of LDL and HDL, resulting in smaller, denser LDL and HDL particles.[158] This CETP-mediated exchange is accelerated in the presence of excess TGs,[166] and explains the hypertriglyceridaemia, reduced HDL and small dense LDL particles commonly observed in diabetes, and Type 2 diabetes, in particular, (Figure 3.6).[158]

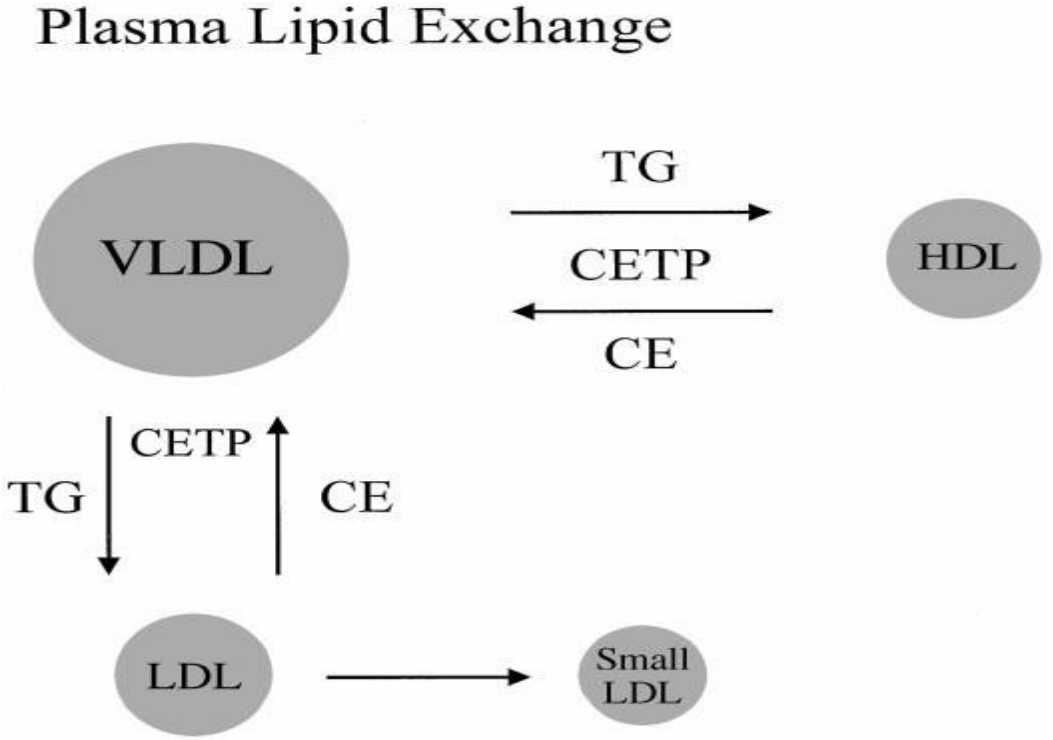


Figure 3.6: Plasma lipid exchange. CETP will exchange VLDL triglyceride for cholesteryl ester in the core of LDL and HDL. The net effect is a decrease in size and an increase in density of both LDL and HDL (Adapted from Goldberg [158]).

Triglyceride levels also correlate with the degree of glycaemic control in patients with Type 1 diabetes.[167] The absolute insulin deficiency in Type 1 diabetes patients presenting with ketoacidosis leads to hypertriglyceridaemia.[168] Additionally, treated Type 1 diabetes patients with poor or suboptimal control also present with elevated TGs, similar to their Type 2 diabetic counterparts, due to relative insulin deficiency.[169] Metabolic control after insulin administration, however, rapidly improves TG levels in Type 1 diabetes by normalising LpL activity. In fact, intensely treated Type 1 diabetes patients often have no observed lipid abnormalities, and plasma TGs may even be lower than non-diabetic individuals.[170]

Dyslipidaemia often precedes the overt appearance of Type 2 diabetes by several years, which suggests that dysregulation of lipoprotein metabolism is an early event accompanying insulin resistance and precedes β-cell failure in Type 2 diabetes.[171] It is worth noting that centrally-deposited adipose tissue (visceral fat or intra-abdominal fat) differs considerably in its metabolic activities and clinical consequences compared with other body fat. Excess subcutaneous fat does not appear to contribute significantly to elevated TG levels [172] compared with visceral adiposity.[173] Visceral adipose tissue is also highly vascular and drains directly into the portal vein. Furthermore, the ability of insulin to suppress lipolysis and to re-esterify FFAs is significantly reduced in visceral adipocytes.[174] In summary, patients with diabetes and/or individuals who are overweight/obese have an increased risk of suffering from dyslipidaemia.

3.4.4 Oxidative stress

While the exact mechanisms by which oxidative stress contributes to diabetic retinopathy remain elusive, it is thought that chronic hyperglycaemia leads to the overproduction of free radicals and that hyperglycaemia also impairs the endogenous antioxidant defence system,[175] rendering the affected cells and tissues (*i.e.* lipids, protein and de-oxy ribonucleic acid (DNA) constituents) more susceptible to oxidative damage.[176] Under normal physiological conditions, ROS are detoxified by their interaction with various reducing and sequestering agents. However, when the body becomes overwhelmed (e.g. by hyperglycaemia), oxidative stress ensues.

Reactive oxygen species can be defined as intermediate oxygen-carrying metabolites with or without an unpaired electron, (*i.e.* oxygen-centered free radicals such as superoxide anions, hydroxyl radicals); and non-radicals (such as hydrogen peroxide,

and singlet oxygen).[177] Free radicals can either donate an electron to or accept an electron from, therefore, behave as oxidants or reductants.[178] Radicals and non-radicals are both capable of oxidising other components, turning them into free radicals, thereby, causing a chain reaction that can lead to the formation of numerous new radicals.[179, 180]

3.4.4.1 Overproduction of reactive oxygen species

Sustained hyperglycaemia and increased chronic local oxidative stress disrupts retinal metabolism and accelerates premature endothelial cell apoptosis in both Type 1 and Type 2 diabetic retinopathy.[181-183] There is also evidence to suggest that oxidative damage occurs in non-vascular retinal cells (Muller, bipolar, amacrine, ganglion, and photoreceptor cells) in the early stages of diabetic retinopathy.[184-186] Glial cells normally produce large amounts of glutathione (GSH), whereas, hyperglycaemia induces depletion of GSH within these cells, resulting in impaired glutamate metabolism.[187] This culminates in the release of inflammatory substances by glial cells which damage their close structural relationship with blood vessels.[188] These pathogenic changes are linked to increased vascular permeability and resultant diabetic macular oedema.

Additionally, Muller cells stimulated by hyperglycaemia increase ROS production which amplifies AGE formation, contributing directly to neuronal cell apoptosis.[189,] Capillary occlusion and retinal ischaemia increase expression and release of growth factors (*i.e.* VEGF) and cytokines (discussed in more detail in section 3.4.5). Vascular endothelial growth factor increases vascular permeability and also favours the growth of abnormal vessels.[190] Pathological angiogenesis driven by VEGF originating from pericytes, retinal ganglion and glial cells has been implicated in irreversible vision

loss.[190]

3.4.4.2 Role of antioxidant defence system

The balance between oxidation and anti-oxidation is believed to be critical in maintaining healthy biological systems. The endogenous antioxidant defence mechanisms include enzymatic and non-enzymatic antioxidants. The primary endogenous antioxidant enzymes are catalase (CAT), glutathione peroxide (GPx) and superoxide dismutase (SOD).[177] Non-enzymatic antioxidants include vitamin E (α-tocopherol), β-carotene, GSH compound and lipoic acid.[177] The endogenous antioxidant system is supported by smaller exogenous antioxidant molecules derived principally from a diet rich in fruit, vegetables and grains, a diet which includes vitamins C and E, carotenoids (β-carotene, lutein, zeaxanthin, *meso*-zeaxanthin) and polyphenols.[177] The synergistic action of several ocular antioxidants is what protects the eye most effectively from oxidative damage.[180] While ROS has been linked to diabetic complications, antioxidants have shown promise as a possible therapy for the prevention and treatment of the disease (reviewed in more detail in chapters four and six).

3.4.5 Inflammation

Inflammation is common in many chronic diseases, including diabetes.[191] In Type 1 diabetes islet inflammation is thought to be a local phenomenon driven by a focal autoimmune attack on islet antigens, whereas, in Type 2 diabetes activation of inflammation results from systemic features such as obesity and insulin resistance.[192] The comorbidities of hypertension, dyslipidaemia, insulin resistance, obesity, (metabolic correlates commonly associated with Type 2 diabetes), have been incriminated in the maintenance and exacerbation of these inflammatory reactions.[192]

Ultimately, inflammatory mediators activate a series of receptors and transcription factors, such as nuclear factor Kappa B (NF-*k*B), which leads to impaired insulin signalling in insulin-sensitive tissues, systemic endothelial dysfunction, and altered vascular flow.[193] Figure 3.7 outlines the association between inflammatory mediators and anatomical changes to retinal tissue in diabetic retinopathy.[194] The role of inflammation in diabetes mellitus will be reviewed in chapter six.

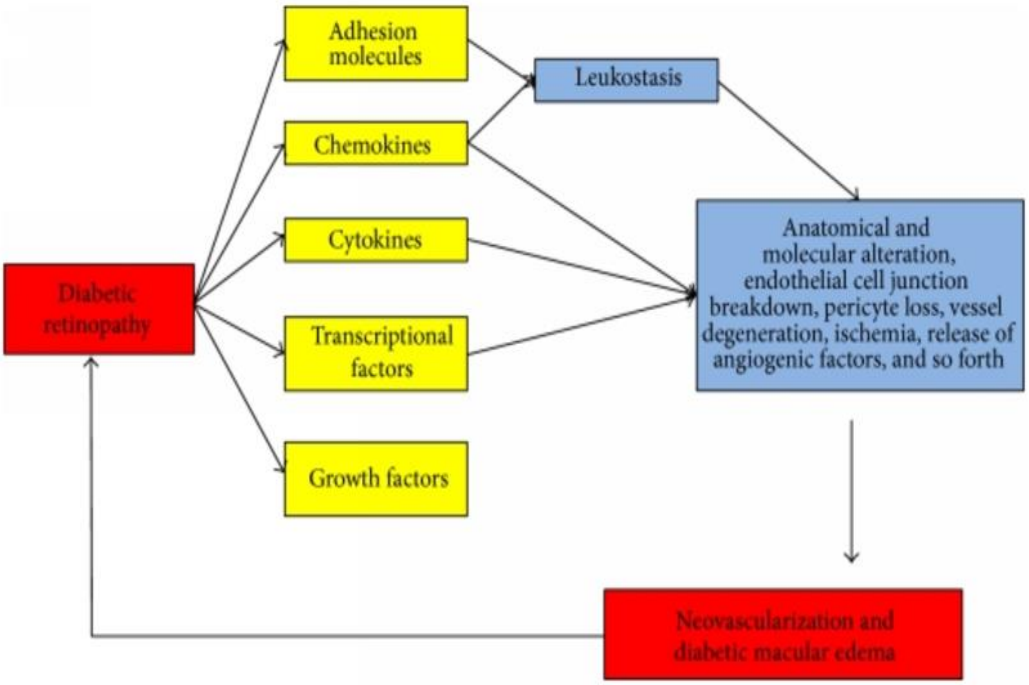


Figure 3.7: Role of vitreous mediators in diabetic retinopathy progression. In diabetic retinopathy, several inflammatory vitreous mediators are upregulated and induce anatomical changes in the retinal tissue. The structural changes enhance retinal tissue degeneration and mediate pathogenesis of diabetic retinopathy (Adapted from Semeraro et al,[194]).

3.4.5.1 Adipose tissue and inflammation

Adipose tissue also contributes importantly to the inflammatory process in

overweight/obese subjects.[195] Pro-inflammatory and pro-coagulant mediators released by stressed adipose tissue have both local and systemic effects on metabolism and vascular function.[192] Adipose tissue from obese individuals contains activated macrophages that together with adipocytes produce inflammatory adipokines such as TNF-α, IL-6, and molecules that may contribute to insulin resistance such as FFAs, TNF-α, and resistin.[196] Overweight/obesity-induced inflammation in association with diabetes mellitus and MP will be discussed in chapters six, seven and .eight

3.4.6 Retinal neurodegeneration

Diabetic retinopathy has traditionally been described as a microvascular disease of the retina, however, more recent research suggests that retinal neurodegeneration is an early event in its pathogenesis. Biochemical and histopathological changes, characteristic of retinal neurodegeneration have been observed in diabetic patients before any visible changes in the retina.[197-199] It is now widely accepted that structural and functional damage to nonvascular cells (ganglion cells, glial and microglial cells) contribute to the pathogenesis of diabetic retinopathy.[135, 200, 201]Important neuroprotective factors include somatostatin (SST) and PEDF. Neurotoxic factors involve elevated levels of extracellular glutamate, oxidative stress and overexpression of the renin angiotensin system (RAS).[202, 203] The balance between neurotoxic and neuroprotective factors is what determines the presence of retinal neurodegeneration in the diabetic retina.

Retinal neurodegeneration has been identified in experimental models on rodent rats at the very early phase of diabetic retinopathy. Rats with streptozotocin (STZ) induced diabetes exhibited a high rate of apoptosis in the neuroretina within one month after inducing diabetes, without any significant apoptosis in endothelial cells.[204] Apoptosis

and glial activation have also been shown to occur in the retina of diabetic patients, before microvascular abnormalities.[197] Retinal ganglion cells appear to be the earliest cells affected and have the highest rate of apoptosis,[205] however, cell death has also been observed in the outer nuclear layer (photoreceptors),[200] and the RPE.[206] Neuroretinal damage produces functional abnormalities early in the course of the disease, as demonstrated by abnormal responses of oscillatory potentials in electroretinograms (ERGs), findings which reflect malfunctioning of inner retinal layers.[201]

It is now believed that carotenoids, lutein, zeaxanthin, and *meso*-zeaxanthin may prevent and protect against the development of diabetic retinopathy via neuroprotective properties, however, the molecular basis for these effects remain unknown. The neuroprotective properties of these carotenoids will be explored in more detail in chapter four.

3.5 Current treatment options for diabetic retinopathy

Diabetic retinopathy remains one of the most debilitating complications of diabetes. Both neuronal and vascular retinal damage occurs as a result of chronic hyperglycaemia, and this damage is progressive, however, most patients remain asymptomatic until very late stages of the disease. Regular screening is, therefore, essential. While there is no cure for diabetic retinopathy, timely treatment may be effective in delaying or reducing vision loss and remains the gold standard for ameliorating blindness due to diabetic retinopathy. Clinically significant macular oedema is the most common cause of moderate vision loss (< 6/12 vision) in all types of diabetic retinopathy. The majority of severe vision loss (< 6/60 vision) in diabetic retinopathy is the result of complications from proliferative diabetic retinopathy, either

from a vitreous haemorrhage, retinal detachment, or neovascular glaucoma. It is recommended that patients with Type 1 and Type 2 diabetes have an initial dilated and comprehensive eye examination by an ophthalmologist or optometrist within 3–5 years after onset of diabetes for Type 1 diabetes and yearly after diagnosis for Type 2 diabetes.[136] Clinical judgment should, however, be used when applying these recommendations to individual patients.[25]

Primary prevention of diabetic retinopathy involves strict control of blood sugars,[145] blood pressure,[156] and lipids.[207] Secondary interventions for diabetic retinopathy include pan-retinal photocoagulation,[208] focal laser photocoagulation,[209] and surgical vitrectomy.[13] More recently, intravitreal anti-VEGF injections have demonstrated their superiority to laser therapy in reducing vision loss and improving rates of vision gain in eyes with diabetic macular oedema.[210] Anti-VEGF therapy has also been shown to be highly effective in regressing retinal neovascularisation in eyes with proliferative diabetic retinopathy.[211] This form of treatment may, however, not be optimal for patients who cannot comply with monthly follow-ups and the repeated intravitreal injections necessary for adequate treatment and prevention of proliferative diabetic retinopathy. Furthermore, intravitreal injections are invasive, increasing the likelihood of local complications including uveitis, cataract, retinal detachment, and endophthalmitis.[212] While intravitreal injections are very effective at preventing vision loss and often result in a visual gain for patients with both diabetic macular oedema and proliferative diabetic retinopathy, there is a substantial proportion of eyes (approximately 40-50%) that do not respond fully to anti-VEGF treatment.[213]

Improvements in understanding the pathogenesis of diabetic retinopathy and an increase in diagnostic techniques, including imaging, opens up the possibility of earlier diagnosis of diabetic retinopathy and will, therefore, optimise efforts toward more precise management of the disease. While Type 1 diabetes patients tend to be diagnosed promptly, patients with Type 2 diabetes can be insulin resistant for many years prior to diagnosis, and can often present with signs of diabetic retinopathy at the time of diagnosis.[25] Lifestyle modifications including an increase in physical activity and a healthy diet (*i.e.* diet of fruit, vegetables, legumes), offer potential to manage diabetes after onset and can even prevent Type 2 diabetes development.[214] Early detection and education on lifestyle behaviours broaden therapeutic objectives beyond direct suppression of pathological vascular changes.

3.6.2 The role of macular pigment in diabetes

While the Age-Related Eye Disease Study 2 (AREDS2) has established a clear clinical benefit for dietary carotenoids (lutein and zeaxanthin) supplementation in patients with AMD,[5] their role in another retinal disease putatively associated with oxidative stress and inflammation (*i.e.* diabetes) remains comparatively unexplored. Current treatment paradigms for diabetic retinopathy (laser photocoagulation, intravitreal injections and vitreoretinal surgery) focus on the treatment of advanced disease, often after permanent damage has ensued. These treatments are also invasive, expensive and need to be repeated at frequent intervals. An important conceptual advance in recent research has been the recognition that diabetic retinopathy is a disease of the neurovascular unit, with multiple interdependent cell types contributing to the dysfunction of the retina. Therefore, new therapeutic approaches should adopt a more holistic view of how diabetes affects the retina. Newer treatments that are preventative or address early pathology are more appealing. Researchers are now beginning to look

at the beneficial effects of lutein, zeaxanthin, and *meso*-zeaxanthin in protecting ocular tissues and cells, including retinal neurons, and on the prevention of diabetic retinopathy which has been studied in a variety of experimental animal models [14, 186, 215] and human studies.[15, 21] There is increasing evidence that MP levels can be augmented through increased intake of dietary lutein and zeaxanthin in both healthy and diseased retinas,[5, 216-218] suggesting the possibility that therapeutic intervention in the form of dietary modification or nutritional supplementation may modulate the risk of diseases with a relative lack of MP.[5, 47] The putative protective role of lutein, zeaxanthin and *meso*-zeaxanthin in the pathogenesis of diabetic retinopathy will now be explored in chapter four.

4. MACULAR PIGMENT AND DIABETES MELLITUS

4.1 Background

The human macula uniquely concentrates three hydroxyl-carotenoids: lutein, zeaxanthin, and *meso*-zeaxanthin, collectively known as MP.[46] Lutein and zeaxanthin cannot be produced endogenously in humans, therefore, these carotenoids must be obtained from dietary sources such as green leafy vegetables and bright coloured fruits,[22] while *meso*-zeaxanthin is believed to be mainly formed at the macula through a poorly understood process of bioconversion.[219] Although, *meso*-zeaxanthin has been found in specific dietary sources such as turtle, shrimp and fish skin.[220] While there are many hundreds of carotenoids present in the plant world, the primate macula contains only these three.

The anatomical location of MP has generated a lot of interest in this pigment's role in vision and macular health. Macular pigment is highly concentrated in the central retina (the macula) and concentrations decrease nearly 100-fold with increasing retinal eccentricity.[44] While not considered to be essential micronutrients, MP's constituent carotenoids have powerful antioxidant, anti-inflammatory and neuroprotective properties [2, 4, 221, 222] and these phytonutrients have been studied extensively. Epidemiological studies and large-scale clinical trials, such as AREDS2, have brought attention to the functional benefits of these carotenoids in the prevention of retinal diseases such as AMD.[5, 218] The fact that MP is modifiable, through diet and/or supplementation, means that ongoing research into their role in the prevention or progression of AMD is important. Researchers are now beginning to focus on the relationship between MP and diabetes mellitus, a condition similarly known to cause oxidative damage and inflammation in the retina.[9, 10] Studies have shown that MP is

lower in diabetes, [6, 9] however, the causal mechanisms and metabolic perturbations associated with lower MP in diabetes have yet to be elucidated. While all humans appear to have some quantity of MP within their retina, foveal concentrations tend to vary quite dramatically.[223,224] The basic science and clinical research underlying the assessment of MP and recommendations for nutritional interventions against diabetic eye disease are under-acknowledged by practitioners and vision researchers alike. This chapter will explore the structural and functional benefits of MP in the eye, with a particular focus on MP and diabetic retinopathy.

4.2 Location of macular pigment

The yellow colouration of the macula is attributable to the presence of lutein, zeaxanthin and *meso*-zeaxanthin.[44] The ratio of lutein to zeaxanthin to *meso*-zeaxanthin is 1:1:1 in the centre of the fovea.[1] Near the fovea, there is twice as much zeaxanthin and *meso*-zeaxanthin as lutein, and in the peripheral retina, this relationship is reversed.[46, 60] Xanthophylls are highly concentrated within the photoreceptor axons of the Henle nerve fibre layer centrally, where the inner retinal layers are displaced.[43, 44] In the parafoveal region they are located in the inner and outer plexiform layers of the photoreceptors.[43, 44] At 7 degrees retinal eccentricity, it is believed that these carotenoids become optically undetectable [60] (Figure 4.1).

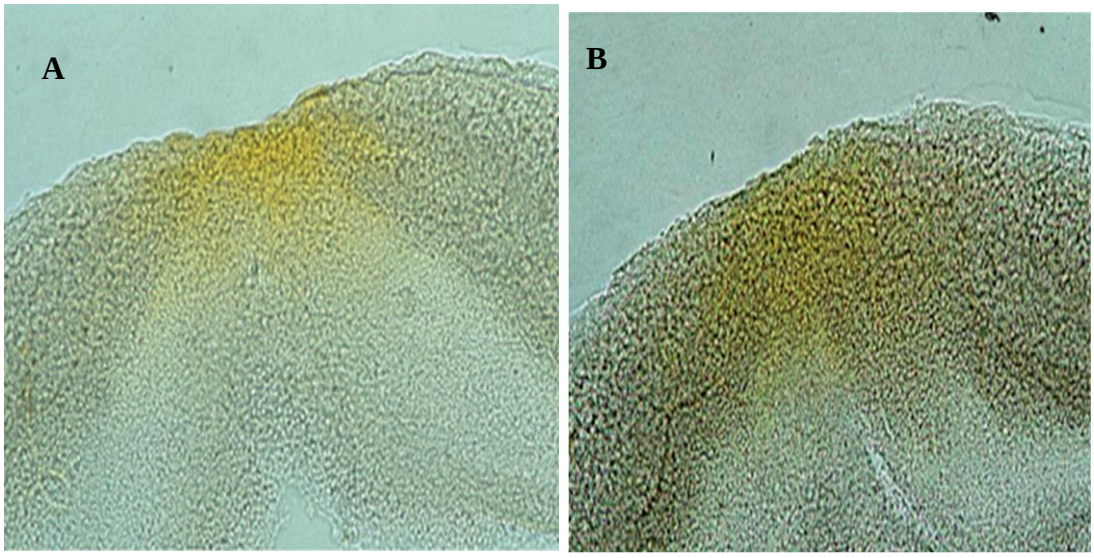


Figure 4.1: Histology of human MP illustrating the spatial profile and pre-receptoral location of MP. The main location of MP is in the layer of the fibres of Henle in the fovea (A) and in the inner nuclear layer at the parafoveal site (B) (Modified from Trieschmann et al,[43]).

4.3 Carotenoid chemistry

The MP of primates can be traced to its dietary origins. Around 700 carotenoids exist in nature, however, only 40 to 50 carotenoids are present in a typical Western diet,[45, 225] of which 15 to 30 enter the human bloodstream.[45, 225] The most prominent plasma carotenoids include lycopene, α-carotene, β-carotene, lutein and zeaxanthin.[45] The carotenoid group include phytochemicals with the basic structure C_{40} H_{56}. These compounds act as antioxidants as they contain several double bonds and react with ROS to scavenge free radicals. Carotenoids are classified as carotenes if they are exclusively hydrocarbons, composed of only carbon and hydrogen, and are classified as oxo-carotenoids (xanthophylls) when they carry at least one oxygen atom.[226-228] Xanthophylls and their hydroxyl (-OH) functional groups permit lutein, zeaxanthin and *meso*-zeaxanthin to cross both the blood-ocular and blood-brain barriers. Carotenes β-carotene and lycopene contain only carbon and hydrogen atoms and do

not cross these barriers.[229] Lutein, zeaxanthin and *meso*-zeaxanthin are the only carotenoids to ultimately reach the human retina.[45, 46] Figure 4.2 shows the chemical structure of lutein, a hydroxyl-carotenoid ($C_{40}H_{56}O_2$).[230]

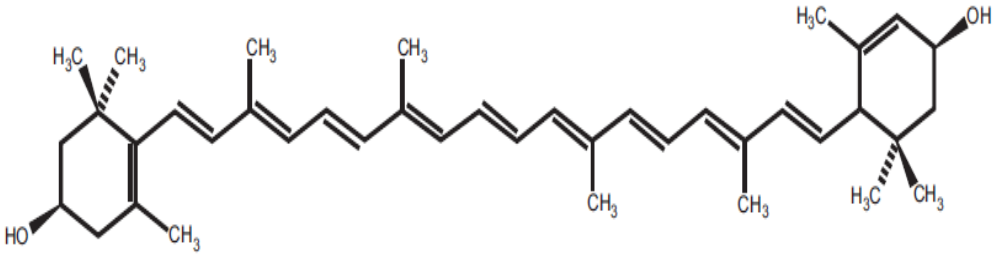


Figure 4.2: Chemical structure of lutein ($C_{40}H_{56}O_2$); lutein contains double bonds that scavenge ROS.[230]

Xanthophylls are a large group of plant pigments responsible for the colour of brightly coloured fruit and vegetables. Lutein is found in the highest concentrations in dark green leafy vegetables (spinach, kale, collard greens and others).[22] Zeaxanthin is the major carotenoid found in corn, orange peppers, oranges and tangerines.[22] The highest molar percentage of lutein and zeaxanthin are found in egg yolk and maize.[22] *Meso*-zeaxanthin, was previously thought to be undetectable in the human liver or plasma and thought to be generated solely at the macula following a biochemical transformation of lutein.[1, 219] The 3:1 ratio of lutein to zeaxanthin in plasma and the 2:1 ratio in the fovea support this theory of conversion.[1] *Meso*-zeaxanthin, however, has more recently been found in trace amounts in blood plasma,[231] and has been identified in some less commonly consumed foods including fish (e.g. salmon and trout), shrimp, and turtle.[220, 232] Supplementation trials have also demonstrated a significant increase in MP's levels after oral supplementation with *meso*-zeaxanthin,

suggesting that it can be absorbed after oral administration and transported to the macula.[233] *Meso*-zeaxanthin accounts for about one-third of total MP at the fovea.

4.3.1 Stereochemistry of macular carotenoids

Macular pigment was shown in 1985 by Bone et al,[234] and later by Handleman et al,[235] using high-performance liquid chromatography (HPLC) to be composed of two chromatographically separable components, namely lutein and zeaxanthin. The lutein component of MP consists of a single stereoisomer of lutein [(3R, 3′R, 6′R)- β, ε-carotene-3,3′-diol], whereas, the zeaxanthin component consists of three possible stereoisomers which include dietary zeaxanthin itself or RR-zeaxanthin [(3R,3′R)-β,β- carotene-3,3′- diol], SS-zeaxanthin [(3S,3′S)- β, β- carotene-3,3′-diol] (found only in trace amounts), and *meso*- zeaxanthin [(3R,3′S)- β, β- carotene-3,3′-diol].[46, 60, 234] Macular pigment refers to the accumulation at the macula of a single isomer of lutein and 3 stereoisomers of zeaxanthin (RRZ, *meso*-zeaxanthin, and SSZ).[44, 46] The hydroxyl group at the C-3′ position in lutein (3R, 3′R, 6′R- β, ε- carotene-3,3′-diol) is configured exactly opposite to that of zeaxanthin (3R, 3′R-β, β -carotene-3,3′-diol), while the C-3 and C-3′ hydroxyl groups in *meso*-zeaxanthin (3R, 3′S-β, β, -carotene-3,3′-diol) are positioned identically to lutein.[226] These hydroxyl groups, one at each side of the molecule, appear to control the biological function of xanthophylls.[219] The double-bond in lutein at the 4′, 5′ position is shifted to the 5′, 6′ position in zeaxanthin and *meso*-zeaxanthin.[226] Additionally, there is an extra conjugated double bond in zeaxanthin and *meso*-zeaxanthin which makes them more stable and better antioxidants than lutein.[236,237,1] Zeaxanthin is superior in preventing lipid peroxidation induced by ultraviolet (UV) light,[238] while *meso*-zeaxanthin has a greater capability of quenching oxygen radicals than lutein, although, lutein has a better filtering efficacy.[239] The functional differences of these carotenoids appear to correlate with

their spatial distribution in the retina. Zeaxanthin and *meso*-zeaxanthin predominate in the centre of the retina where cone density is highest and risk of oxidative damage is greatest.[235, 237] The combination of all three carotenoids and their essential functions, however, is what reduces oxidative stress in both normal and diseased retinas. Figure 4.3 shows the stereochemistry of all three macular carotenoids.[226]

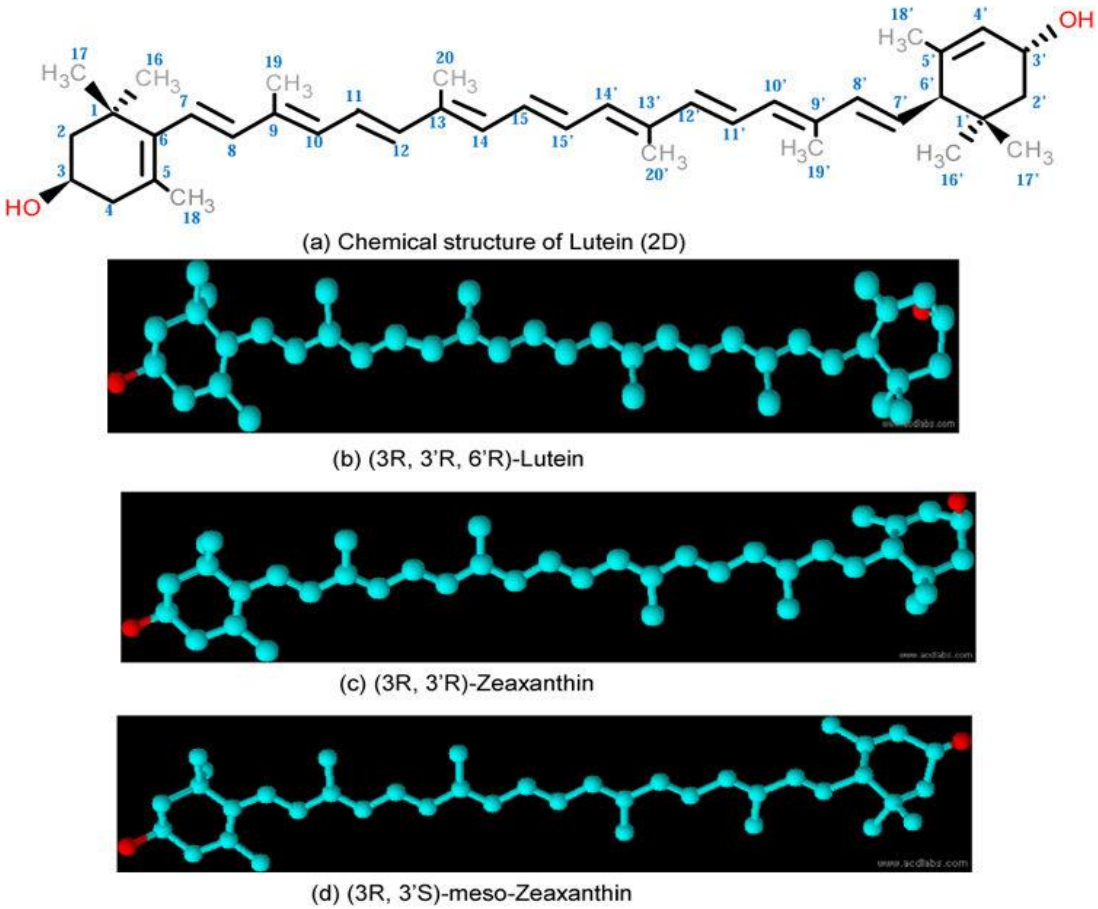


Figure 4.3: Chemical structure of macular pigment carotenoids. (a),(b) lutein; (c) zeaxanthin; (d) meso-zeaxanthin (Adapted from Bernstein et al,[226]).

4.4 Functional properties of macular pigment

The retina is an ideal environment for the generation of ROS for several reasons; 1) the retina is subject to high levels of cumulative irradiation; 2) the membranes of the outer segments of photoreceptors have high concentrations of polyunsaturated fatty

acids (PUFAs) which are susceptible to photo-oxidation; [240] 3) oxygen consumption by the retina is much greater than by any other tissue and the outer retina has a high oxygen tension (70 mmHg), almost that of arterial blood; 4) furthermore, the process of phagocytosis by the RPE itself creates oxidative stress.[241] Therefore, a rich oxygen supply, combined with high-energy short-wavelength light stimulation, and a vulnerable substrate creates ideal conditions for oxidative damage.[242] Some of the major proposed functions of these ocular carotenoids include blue light filtration effects, antioxidant, anti-inflammatory and neuroprotective properties, and are discussed in more detail herein.[2, 221, 222, 243, 244]

4.4.1 Macular pigment as a blue light filter

Macular pigment reduces sensitivity of the macular region to short-wavelength light by acting as a broadband filter.[245] Most UVC (100-200 nm) radiation is absorbed by the atmosphere; while UVB (290-320nm) and UVA (320-400nm) radiation are absorbed by the cornea and crystalline lens. Slightly longer-wavelength light (400-500 nm) is for the most part absorbed by MP, which has a peak absorbance of 460nm.[44, 246] Absorption by MP occurs before light reaches the photoreceptors,[44] therefore, penetration of damaging short-wavelength light is minimised. While macular xanthophylls reach their highest concentrations in the centre of the fovea, these carotenoids are also highly distributed throughout the photoreceptor cell. Therefore, each photoreceptor screens other photoreceptors as well as itself because of the lateral course of the axons.[44]

Blue light being a shorter wavelength has more energy and is, therefore, potentially more damaging to the eye. Chronic exposure to radiation (400-520 nm) can cause damage to both the intraocular lens and the retina through photo-oxidative reactions.[247]

Beyond the first three years of life yellow chromophores develop in the crystalline lens which are capable of absorbing UV and short-wavelength light.[248] Because of these chromophores the absorbed energy is safely dissipated and the lens and retina are protected against photo-oxidative damage.[249] With advancing age, however, these chromophores become modified and phototoxic. Absorption of UV and short-wavelength visible light by a phototoxic chromophore causes the chromophore to become excited, firstly to a singlet state, then a triplet state, and from a triplet state free radicals and ROS are produced (e.g. singlet oxygen and superoxide), free radicals which can damage ocular tissue (reviewed by Roberts & Dennison [250]), (Figure 4.4).

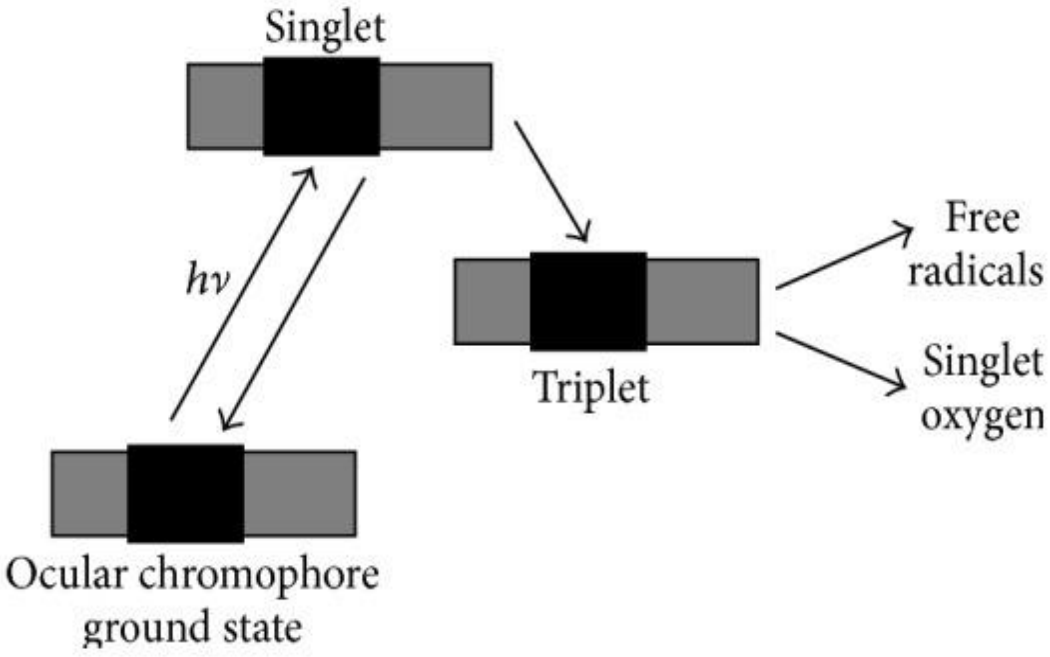


Figure 4.4: Photo-oxidation (Adapted from Roberts & Dennison [250]).

Exposure to UV and short-wavelength blue light is particularly hazardous after the age of 40 because there is an increase in light-absorbing endogenous phototoxic chromophores, that efficiently produce singlet oxygen and other ROS. Chromophores which produce free radicals and singlet oxygen upon photoexcitation are known as photosensitisers. A single molecule of a photosensitiser is capable of producing

numerous free radicals and singlet oxygen molecules as long as it is recycled and photoexcited by subsequent photons. It is thought that increased levels of ROS and the concomitant decrease in the production of antioxidants and antioxidant enzymes in the lens leads to DNA damage and cross-linking of proteins, resulting in cataract formation.[251]

The continual exposure of the retina to incident light, in addition to the high concentrations of oxygen, and the presence of photosensitisers, such as vitamin A derivatives, rhodopsin, cone opsins, lipofuscin and melanin, make the outer retina inherently susceptible to photo-oxidative damage. Damage induced by chronic phototoxic reactions over many years has been implicated in the aetiology of AMD.[252] Blue light-induced generation of ROS by the lipofuscin chromophore is toxic to mitochondria and is known to initiate apoptosis in the RPE.[253,254] Furthermore, it has been shown that blue light damage is proportional to the amount of light received and the amount of lipofuscin within RPE cells.[255] Chromophores in photoreceptor outer segments (rhodopsin and cone opsins) are also susceptible to oxidative damage. While most UV light is absorbed by the cornea and lens, some fraction of blue light radiation reaches the retina and may activate potent retinal photosensitisers. Blue light absorption by MP can, therefore, indirectly be considered an antioxidant action because it prevents blue light from generating ROS which can potentially damage photoreceptor cells.[256]

4.4.1.1 Blue light-filtering effects of MP in diabetes

Several studies have investigated the protective effects of lutein and zeaxanthin administration against light-induced damage in experimental animal models. Evidence suggests that zeaxanthin can protect photoreceptors against light-induced apoptosis in

quail (the retinae of which, like those of primates, selectively accumulate lutein and zeaxanthin).[257] Oral supplementation with zeaxanthin was found to decrease photoreceptor cell death and activation of microglia in this study, providing experimental evidence that xanthophyll carotenoids can protect photoreceptor cells *in vivo*.[257] The effects of lutein in light-induced retinal degeneration has also been investigated in STZ induced diabetic murine models.[186] Functional abnormalities secondary to diabetes-induced oxidative stress, (*i.e.* reduction in ERG a and b-wave amplitudes), were observed before histopathological changes in diabetic retinopathy.[186] These changes were, however, successfully restored following administration of lutein, suggesting that carotenoids have the potential to normalise diabetes-induced early functional modifications in the retina.[186] The beneficial effects of MP on diabetes in experimental animal and human studies will be explored in more detail in chapter six.

4.4.2 Macular pigment as a potent antioxidant

Carotenoids lutein, zeaxanthin and *meso*-zeaxanthin are part of the antioxidant defence system. By nature of their biological structure and function, these phytonutrients help neutralise ROS. Carotenoids interact synergistically with other antioxidants, minerals and vitamins, thereby, protecting the eye against the cumulative effects of damage due to light and oxygen.[45,258,250] There is increasing evidence that higher intakes of fruits and vegetables (*i.e.* carotenoids lutein and zeaxanthin),[22, 259] can slow the progression of age-related cataracts and AMD.[5, 260] The beneficial effects of higher intakes of fruit and vegetables in diabetes and diabetic retinopathy is growing, however, findings are not as definitive.[261]

Oxidative damage is one of the underlying causes of diabetic retinopathy. While free-radicals can occur as normal by-products of cellular metabolism,[262] the generation of ROS increases dramatically in the retina during times of local stress (*i.e.* hyperglycaemia). *De novo* synthesis of MP is not possible in humans, therefore, an important source of theses phytonutrients is a diet which is rich in fruit and vegetables,[22] a diet which may be compromised in diabetes (Type 2 diabetes in particular).[214] Additionally, there is a decline in the production of antioxidants and antioxidant enzymes with age, (reviewed by Liguori et al,[263]). Oxidative stress appears to be caused by both increased production of ROS and/or reductions in antioxidant defences, thereby, resulting in an altered redox state,[264, 265] (reviewed in more detail in chapter six).

In brief, carotenoids lutein, zeaxanthin and *meso*-zeaxanthin are powerful quenchers of singlet molecular oxygen and free radical scavengers.[266-268] The quenching of singlet oxygen is primarily by a physical mechanism rather than a chemical one. The carotenoid molecule accepts the excitation energy from singlet oxygen. The added energy causes excitation of the carotenoid molecule, resulting in a triplet state and this triplet state dissipates energy harmlessly as heat through rotational and vibrational interactions and finally relaxes into a ground state. The structure of the carotenoid remains unchanged in this process.[229, 267] Xanthophylls lutein, zeaxanthin and *meso*-zeaxanthin can also scavenge ROS (free radicals such as hydroxyl and peroxyl radicals). The free radical can either obtain its missing electron from the electron-rich molecule of the carotenoid or the free radical adds itself to the carotenoid molecule to pair its single electron, thus forming a covalent bond.[229, 267] Either way, the electron-rich structure of the carotenoid molecule attracts free radicals and spares cell damage

to lipids, proteins or DNA.[269] Figure 4.5 shows the photochemical mechanisms of protection by lutein and zeaxanthin.[250]

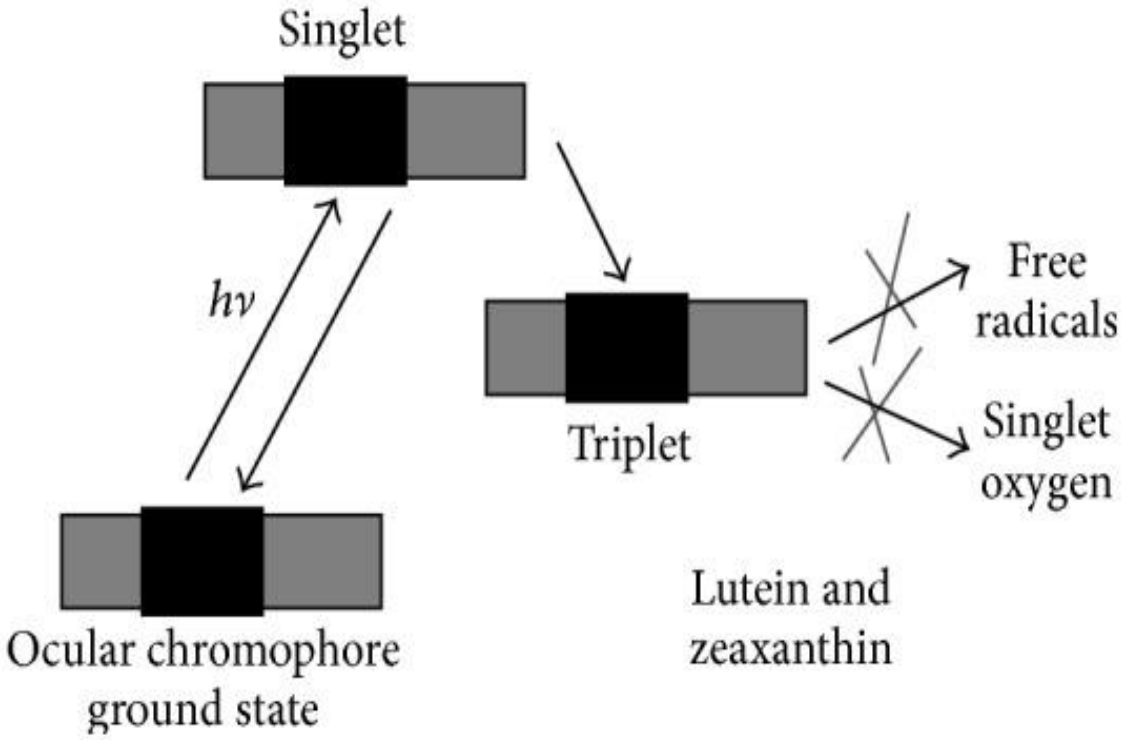


Figure 4.5: Photochemical mechanisms of protection (Adapted from Roberts & Dennison [250]).

Carotenoids have a ready supply of available electrons that enable them to scavenge ROS and attenuate oxidative injury. Zeaxanthin, (with 11 conjugated double bonds), has a higher ability to quench singlet oxygen than lutein (with 10 conjugated double bonds).[229,270] *Meso*-zeaxanthin, in conjunction with its binding protein, however, is even more effective.[271] *Meso*-zeaxanthin differs from zeaxanthin in the spatial orientation of the hydroxyl group on the C3 chiral position, but the conjugated systems are the same. The order of efficiency of the macular carotenoids in quenching singlet oxygen is as follows: lutein < zeaxanthin < *meso*-zeaxanthin < all three combined.[272] A mixture of lutein: zeaxanthin: *meso*-zeaxanthin in a ratio of 1:1:1 however, has

shown to be the most effective in quenching singlet oxygen.[272]

4.4.2.1 Antioxidant function of MP in diabetes

Evidence is emerging which suggests that oxidative damage occurs in retinal vascular cells (endothelial and pericyte cells) as well as non-vascular cells (Muller cells, photoreceptor, bipolar and ganglion cells) in the early stages of diabetic retinopathy.[184] Hydroxyl-carotenoids, lutein, zeaxanthin, and *meso*-zeaxanthin readily supply available electrons that enable these carotenoids to scavenge ROS. Although the evidence exploring MP and diabetes is relatively sparse, it has been shown that plasma concentrations of lutein and zeaxanthin are lower in diabetes patients with non-proliferative diabetic retinopathy compared with normal controls and that supplementation with these carotenoids can improve VA, contrast sensitivity and macular oedema in patients with diabetic retinopathy.[15] The putative protective effects of MP in diabetic retinopathy in human and animal studies will be explored in more detail in chapter six.

4.4.3 Neuroprotective and anti-inflammatory properties of MP

The presence of lutein and zeaxanthin in substantial concentrations in the primary visual cortex [273] and the finding of better dark-adapted cone sensitivities in association with higher MP [274] combine to suggest a key role for MP in ocular and neurophysiologic health. The retina is part of the central nervous system and once damaged regenerates poorly, if at all. The majority of studies investigating the effects of MP augmentation in ocular disease, including AMD and diabetes, have reported beneficial effects on vision [21, 218] findings which suggest a neuroprotective effect of these carotenoids. While the exact mechanisms are not clear, there are a number of plausible explanations to explain why these phytonutrients improve neural function,

including, enhancement of membrane stability, modulation of synaptic activity and intracellular communication.[256, 275, 276]

The orientation of xanthophyll molecules within the phospholipid bilayers not only protect against oxidative damage but also modulate the physical properties of these membranes.[275] Macular xanthophylls are distributed between the lipid and protein components of membranes.[226] The presence of polar hydroxyl groups at each end of the xanthophyll molecule ensures a roughly perpendicular orientation within the lipid bilayer.[256, 275] The almost vertical orientation of the dipoles provides good conditions for macular xanthophylls to act as a blue light filter.[275] Concomitantly, such an organisation has a direct effect on reducing the penetration of molecular oxygen into the hydrophobic membrane core, thus protecting vulnerable regions against oxidative damage.[256] The high membrane stability and preferential transmembrane orientation in lipid bilayers of photoreceptor outer segments, in addition to their antioxidant properties, maximises their protective action in retinal membranes.[275]

Lutein and zeaxanthin may also play a role in maintaining cell integrity and plasticity, by binding to tubulin.[277] Tubulin has been identified as the major carotenoid binding protein found in abundance in the receptor axon layer of the fovea.[277] Tubulin is also the main structural protein of microtubules.[277, 278] Microtubules form important cytoskeletal structures that play a role in maintaining neuronal morphology, transporting cargo, and scaffolding signalling molecules.[279] Reduced microtubule stability has been observed in neurodegenerative diseases such as Alzheimer's disease and Parkinson's disease [279] and research suggests the macular carotenoids modulate the stability of photoreceptor-axon microtubules, thereby, promoting cell integrity.[278]

Particular attention has also been given to the stimulatory effects exerted by carotenoids on gap junctional communication. While chemical synaptic transmission (via transmitters, neurotransmitters) is the dominant mode of signal transmission, inter-neuronal signal transmission is also mediated by gap junctions. Gap junctions provide low resistance avenues for electrical and/or metabolic communication of coupled cells.[280] Direct coupling through gap junctions has been suggested to mediate communication between neurons and glial cells.[280,281] The regulation of gap junctional communication is complex and the mechanisms related to carotenoid activity are not, however, fully understood. It has been suggested that lutein and zeaxanthin increase processing speed through their facilitative effect on gap junctional communication.[282] In theory, a high density of pigment may improve signal transduction in the visual system.[282] The connection between photoreceptors and the visual cortex may also be strengthened, which could explain improvements in contrast sensitivity function after three months of supplementation with lutein and zeaxanthin in patients with non-proliferative diabetic retinopathy.[15]

There is also growing evidence to suggest that lutein and zeaxanthin may prevent the development of diabetic retinopathy by suppressing ROS induced by inflammation.[14, 283] Reactive oxygen species are considered a strong stimulus for the activation of a number of pro-inflammatory pathways and several intracellular signalling pathways of inflammation are associated with oxidative damage. In the endotoxin-induced uveitis (EIU) mouse model, the innate immune system causes intraocular inflammation which involves the neural retina.[185, 284] One effect of retinal inflammation that impairs visual function is the decreased expression of rhodopsin, an essential protein that is vital for photo-transduction and photoreceptor function. In the

EIU mouse model, ERG showed a decrease in a-wave amplitude and photoreceptor dysfunction.[185, 284-286] It is thought that a reduction of rhodopsin is caused by the upregulation of signal transducer and activator of transcription 3 (STAT3) and activation of inflammatory signals downstream, (*i.e.* IL-6). Once STAT3 activation exceeds a certain threshold, the IL-6-STAT3 pathway enters a vicious cycle, which exacerbates pathological conditions. It was found that the loss of rhodopsin resulted in shorter outer segments and a decrease in photoreceptor cell function.[287] To investigate if lutein can protect visual function by suppressing inflammation the EIU model mice were pre-treated with lutein. Treatment with lutein prevented a reduction in the rhodopsin protein and preserved ERG a-wave, thereby, protecting photoreceptor cell function in the inflamed retina.[230, 285] Lutein's effect on rhodopsin preservation was accompanied by a suppression of STAT3 activation,[230] which also suppressed the IL-6-STAT3 cycle, effectively preventing tissue damage.[230] While the interaction between oxidative stress and inflammation is complex, these findings suggest that lutein also has an anti-inflammatory effect.[230]

The benefits of MP are many and may relate to its blue light filtering effects, antioxidant, anti-inflammatory and neuroprotective properties. The link between hyperglycaemia and neurodegeneration is, however, not fully understood but various cues point to altered inflammation and the activation of retinal microglia as early features of diabetic retinopathy.[288,289, 290] The protective effects of lutein and zeaxanthin in animal and human studies will be reviewed in more detail in chapter six.

4.4.3.1 Polyunsaturated fatty acids

The photoreceptor membranes of both rods and cones contain high quantities of long-chain omega 3 PUFAs. Polyunsaturated fatty acids account for about 50% of the lipid

bilayer of rod outer segment membranes and proteins make up the remaining 50%. Polyunsaturated fatty acids are particularly susceptible to free radical damage because their conjugated double bonds are convenient sources of hydrogen atoms, which contain one electron. The lipid radical combines with oxygen to form lipid peroxyl radicals and lipid peroxides, which can only achieve a steady-state by stealing electrons from other PUFAs, thus, creating a cytotoxic cascade of reactions that consume valuable PUFAs and produce damaged molecules.[291, 292] Docosahexaenoic acid (DHA) is the main omega-3 PUFA in photoreceptors and the most oxidisable fatty acid in the body.[293] Docosahexaenoic acid contains six double bonds so the retina is inherently susceptible to lipid peroxidation. Lipid peroxidation of membrane PUFAs results in loss of membrane function and structural integrity.[294]

Omega-3 fatty acids DHA and eicosapentaenoic acid (EPA) have, however, also been shown to inhibit a wide range of inflammatory mediators involved in the pathophysiology of diabetic retinopathy, as well as, decrease the formation of free radicals and induce the expression of endogenous antioxidant enzymes, (reviewed by Behl & Kotwani [295]). The anti-inflammatory and anti-angiogenic responses of DHA appears to be more potent than EPA [296] and DHA is known to regulate immune function through microglia activation.[292,297] Recent studies suggest that PUFAs and their metabolites could play a significant role in diabetic retinopathy,[298] as both EPA and DHA inhibit the production of both IL-6 and TNF-α,[299, 300] and PUFAs also suppress VEGF production.[301, 302] Lutein, zeaxanthin and *meso*-zeaxanthin are ideally located in the rod outer segment membranes, suggesting a protective role as potent antioxidants for these important lipids.[303,60, 291,304] Interestingly, the effect of supplementation with a multi-component nutritional supplement which included

omega 3 (EPA and DHA) and lutein/zeaxanthin, among other nutrients have been shown to exert positive and beneficial ocular effects in those with diabetic eye disease, (Type 1 and Type 2).[21] This concept will be explored in more detail in chapter six.

4.4.3.2 Vitamin D

Vitamin D is also known to have anti-inflammatory and anti-angiogenic properties.[305] Evidence is accumulating that higher levels of vitamin D may protect against the development of diabetic retinopathy,[306, 307] and that low plasma vitamin D levels are associated with increased severity of diabetic retinopathy.[308] Abnormalities in many systemic inflammation markers (*i.e.* TNF- α, IL-6) have been found in Type 2 diabetes and it has been reported that vitamin D down-regulates the production of these pro-inflammatory cytokines (reviewed by Palomer et al,[309]). Furthermore, the angiogenic effects of vitamin D deficiency have been shown to contribute to vascular damage in a group of young adolescents with Type 1 diabetes.[310] Due to the cross-sectional nature of many of these studies, however, it is not known if vitamin D deficiency is a cause or a consequence of retinopathy. The relationship between vitamin D, MP and Type 2 diabetes will be explored in more detail in chapter seven.

4.5 Absorption, delivery and uptake of carotenoids in the eye

While a diet rich in fruit and vegetables is important for achieving adequate intake of lutein and zeaxanthin (as well as other nutrients), concentrations of carotenoids in the retina to form MP are highly variable and reflect not only dietary consumption but also other factors such as individual efficacy of absorption,[70] cholesterol and lipoprotein status,[18, 71] body composition/bodyfat,[17, 54] and metabolic status,[6] among others. Individual differences in MP may also be influenced by non-dietary factors such as genetics,[72] demographic and lifestyle characteristics. The bioavailability,

process of intestinal absorption, transport, and delivery of these phytonutrients to the retina will now be explored.

4.5.1 Recommended daily intake values for carotenoids

At present there is no official recommended dietary intake level or established physiologically significant cut off points for lutein/zeaxanthin in plasma above which 'protection' or 'prevention' against chronic diseases (*i.e.* AMD, diabetic retinopathy) are ensured. Based on previous research,[259] an intake of 6 mg of lutein and zeaxanthin per day for men and women has been suggested as a dietary target to reduce the risk of AMD.[311] Mean daily intake of lutein and zeaxanthin combined, ranges from 0.8 mg to 4 mg per day, depending on the population studied and the method of dietary assessment used.[22, 312] An Irish study, involving 826 subjects estimated mean lutein/zeaxanthin intakes of 0.6 - 2.4mg/day.[49] The majority of lutein supplements contain in the range of 6 - 25mg/day. Many supplements, however, are often well in excess of the recommended levels of certain minerals and vitamins, with some dosages carrying potential adverse effects. It is worth noting that while lutein is considered a very safe compound, an exceedingly high daily ingestion of lutein (e.g. daily 20 mg supplement of lutein; 4 g of fish oil; and dietary consumption of lutein, which included a broccoli, kale, spinach, and avocado smoothie every morning) was associated with a case of crystalline maculopathy.[313] In this regard, a comprehensive dietary approach to optimise carotenoid intake is preferable and would be a positive first step in maintaining eye health.

4.5.2 Bioavailability, absorption and uptake of macular carotenoids

Many factors can affect the bioavailability and absorption of carotenoids including; 1) food processing (raw, dehydrated, frozen, cooked); 2) meal composition; 3) activity

of digestive enzymes; and 4) the effective uptake of carotenoids by enterocytes.[314] Carotenoid metabolism begins in the stomach. The initial phase of digestion/absorption involves the extraction of carotenoids from the food matrix, followed by their incorporation into lipid droplets, and transfer to mixed micelles.[315,316] The extent to which this happens depends on the quantity and nature of lipids present in the food bolus.[314] Lipids increase the bioavailability of both free and esterified lutein,[317] as they facilitate the extraction of carotenoids from the food matrix by providing a hydrophobic phase in which carotenoids can solubilise. Dietary triglycerides stimulate biliary secretion and consequently micelle production, therefore, can increase carotenoid availability for absorption (reviewed by Desmarchelier & Borel [314]). Dietary fibre, on the other hand, can inhibit lutein and zeaxanthin absorption.[318] It is also known that some micronutrients compete with carotenoids regarding their absorption. The presence of β-carotene can lead to competition for incorporation into mixed micelles and subsequent uptake by enterocytes.[319]

Uptake and absorption of dietary carotenoids in the duodenum is by a facilitated process which utilises scavenger receptor class B member 1 (SR-B1),[320] a cell surface glycoprotein and a member of CD-36 (cluster of differentiation 36) superfamily.[321] Once absorbed carotenoids follow the same route as other newly absorbed lipid molecules (e.g. fatty acids, cholesterol) and are incorporated with them in chylomicrons in the Golgi apparatus before secretion in the lymph (apolipoprotein B dependent route).[314] As some carotenoids are also found in HDL and since the small intestine synthesises HDL, it is hypothesised that a fraction of carotenoids are also secreted into the lymph in HDL (apolipoprotein A dependent route).[322]

4.5.3 Plasma transport and effective uptake of carotenoids

Carotenoids that reach the liver via chylomicrons are either stored there, eliminated in bile, or re-secreted into VLDL to be distributed to peripheral tissues.[314] While carotenoids are detectable in all lipoprotein classes to varying degrees, most hydrophobic carotenoids such as lycopene and β-carotene are transported on LDL, whereas, the more hydrophilic xanthophyll carotenoids, such as lutein and zeaxanthin, are primarily carried by HDL.[19,323,324] The Wisconsin hypo-alpha mutant (WHAM) chicken has very low levels of HDL due to a mutation in the ABCA1 transporter gene.[18] When these chickens are fed a high-lutein diet, lutein levels increase in plasma, heart, and liver, but not in the retina, suggesting that HDL is critical for the delivery of carotenoids to retinal tissue.[18]

Lipoprotein-bound xanthophylls, lutein, zeaxanthin and *meso*-zeaxanthin, accumulate in the macula. To reach the neural retina (Muller cells, receptor axons and retinal ganglion cells) carotenoids cross at the RPE. The RPE is similar to the blood-brain barrier for the retina in that it serves as a cellular and metabolic interface between the retina and the blood supply from the choroid.[325] It has been suggested that lutein and zeaxanthin are preferentially take up by human RPE cells via an SR-B1 dependent mechanism.[326] Differentiated ARPE-19 cells express lipoprotein receptors necessary for lipoprotein delivery (*i.e.* SR-B1, LDL receptor and CD-36).[326] While xanthophylls are mostly associated with HDL in plasma, recent research suggests that zeaxanthin and *meso*-zeaxanthin are more efficiently delivered to ARPE-19 cells via HDL in an SR-B1-dependent process, whereas, lutein is more efficiently delivered via LDL and cell uptake is thought to involve the LDL receptor.[327] It is possible that these mechanisms account for the selective accumulation of zeaxanthin and *meso*-

zeaxanthin over lutein in the central macula.[328]

Specificity and uptake of xanthophylls in specific regions of the retina may ultimately be driven by selective binding proteins such as glutathione S-transferase (GSTP1) for zeaxanthin and steroidogenic acute regulatory protein (a StARD family protein) for lutein.[329] It is thought that tubulin may serve as a secondary high capacity deposition site for carotenoids within the Henle fibre layer.[226, 329] Furthermore, a number of enzymes that metabolise ocular carotenoids have also been identified, including beta-carotene 15,15′ oxygenase-1 (BCO1) which cleaves carotenes, an essential step for generation of vitamin A, and β-carotene 9′,10′ oxygenase-2 (BCO2) which cleaves carotenes and xanthophylls.[330] The process by which carotenoids and their oxidation and degradation products are removed from the retina, however, is less clear. It is known that macular carotenoid levels are remarkably stable even in the face of large fluctuations in plasma levels that might occur with diet and/or supplementation. Moreover, non-human primates placed on carotenoid-deficient diets typically take several years to achieve non-detectable MP.[331] Figure 4.6 provides a brief schematic to describe the current understanding of the whole process of uptake, transport, and accumulation of MP carotenoids in the human retina (adapted from Li et al,[329]).

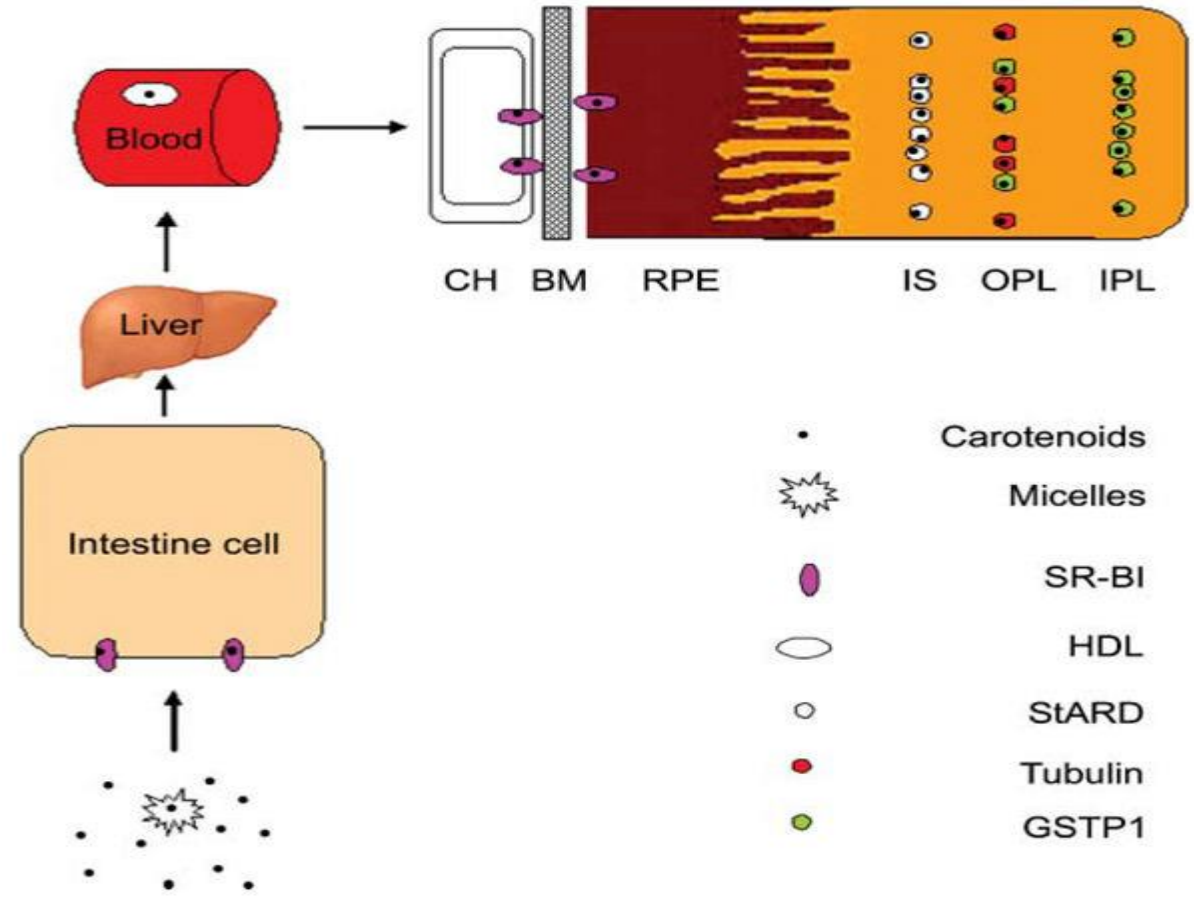


Figure 4.6: Possible pathway for MP carotenoid uptake, transport, and accumulation in the human retina, (Adapted from Li et al, [329]).

Abbreviations: Choroicapillaris (CH); Bruch's membrane (BM); retinal pigment epithelium (RPE); inner segments (IS); outer plexiform layer (OPL); inner plexiform layer (IPL).

4.6 Quantification of macular pigment in clinical practice

The protective effect of MP, if any, can be investigated through the observation of MP levels. From the findings of epidemiological studies and AREDS2, there has been great interest in quantifying carotenoid status in the eye.[5, 332] Macular pigment levels can be augmented with dietary modification,[47] and/or nutritional supplementation,[333] therefore, in measuring MPOD on a wide-scale it may be possible to analyse the effects of MP augmentation on disease process and progression (*i.e.* the effects of MP

augmentation on diabetic retinopathy). It is extremely challenging, however, to measure MP *in vivo* and although several measurement modalities have been proposed, most techniques are only available in a research setting. In humans, measurement of MP *in vivo* is typically categorised into one of two groups: psychophysical (requiring a response from the subject) or objective (requiring minimal input from the subject), and will be discussed herein.

4.6.1 Psychophysical techniques

Psychophysical techniques include colour matching,[9] heterochromatic flicker photometry (HFP),[334] and customised-HFP (c-HFP).[335] One of the more common methods of measuring MP is the HFP technique which is often used as a standard against which other techniques are validated.[336, 337] The HFP method exploits the spectral absorption properties and retinal location of MP. The MPOD is determined by presenting a light stimulus of two alternating wavelengths, a green light (not absorbed by MP) and a blue light (maximally absorbed by MP) and the subject is required to make isoluminance matches.[334] If the colours are alternated at an appropriate frequency and the luminance of the two colours are not perceived to be equal then the stimulus will appear as a flickering light.[338] The radiance of the blue light is adjusted by the subject until the observed flicker is minimised.[334] The procedure is performed centrally, typically at 0.5^0 retinal eccentricity (where MP peaks) and repeated at a parafoveal locus, typically 7^0 (where MP is optically undetectable).[339] The log ratio of the amount of blue light absorbed centrally to that absorbed at a peripheral retinal locus (the reference point) gives a measure of the subject's MPOD. Customised-HFP enhances the HFP technique by optimising the flicker frequency for each individual.[334, 335] The Macular Densitometer™ (a device which uses c-HFP) has been validated by comparing MP measurements to known

biochemical markers (*i.e.* plasma concentrations of MP's constituent carotenoids) and by comparing the data it generates with the *in vitro* spectral absorption curve of the macular carotenoids [339] (Figure 4.7). Importantly, HFP and c-HFP have demonstrated an ability to detect changes in MPOD following supplementation with MP's constituent carotenoids.[333, 340]

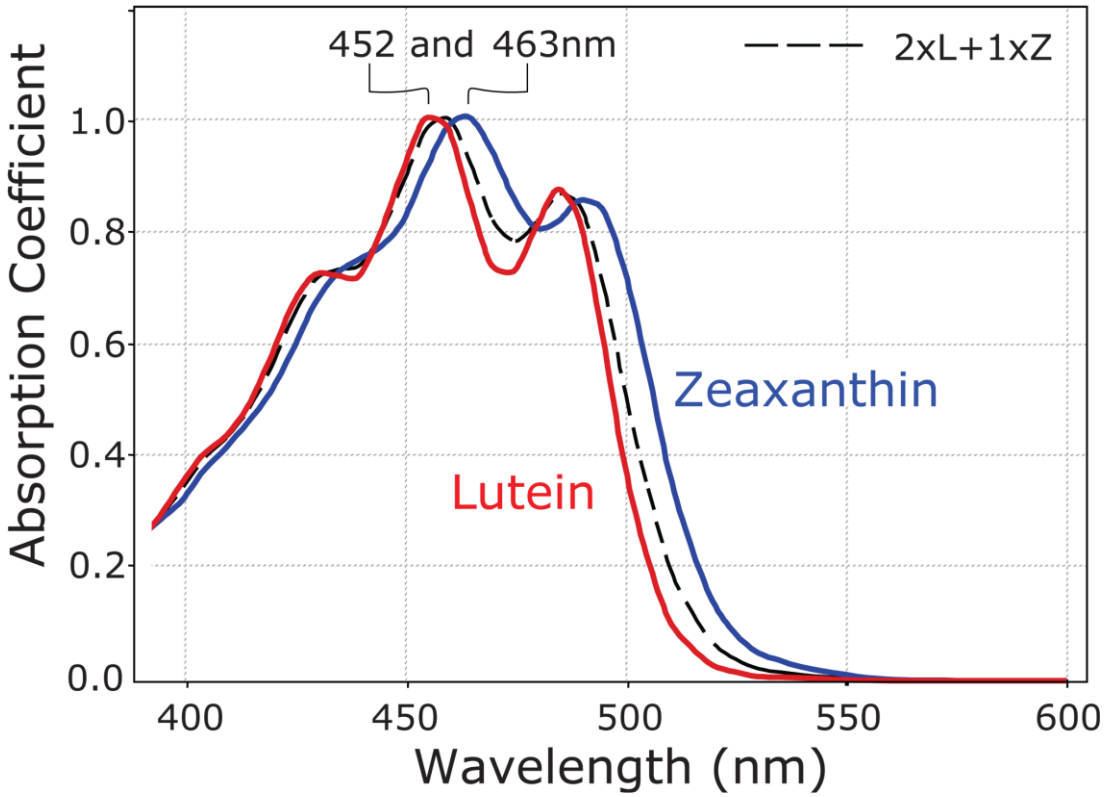


Figure 4.7: Absorption spectra of lutein (red) and zeaxanthin (blue) in olive oil. A mixture of lutein plus zeaxanthin (dashed black line) closely approximates the absorption spectrum of the MP in the living human eye (Adapted from Bernstein et al,[339]).

The HFP measurement relies on several assumptions; 1) the amount of yellowing in the media (e.g. the crystalline lens) would influence the measured MPOD value, however, the reference measure outside of the fovea cancels this effect.[341]

Consequently, varying degrees of crystalline lens absorption does not affect MP measurement and; 2) the peripheral reference locus (typically taken at 7^0 retinal eccentricity) has a negligible level of MP.[339] An advantage of the HFP method is that pupil dilation is not necessary. A drawback of the technique, however, is that it requires significant patient training to produce meaningful results. Additionally, some protocols only take two measurements, centrally and peripherally, therefore, providing no information on the spatial distribution of MP. It is possible, however, to achieve a spatial profile of MPOD by altering the size and/or the eccentric position of the test stimulus. While the extra measurements increase the duration of the test, it produces a curve that can be used to describe the change in MPOD with increasing eccentricity from the central fovea [342] (Figure 4.8).

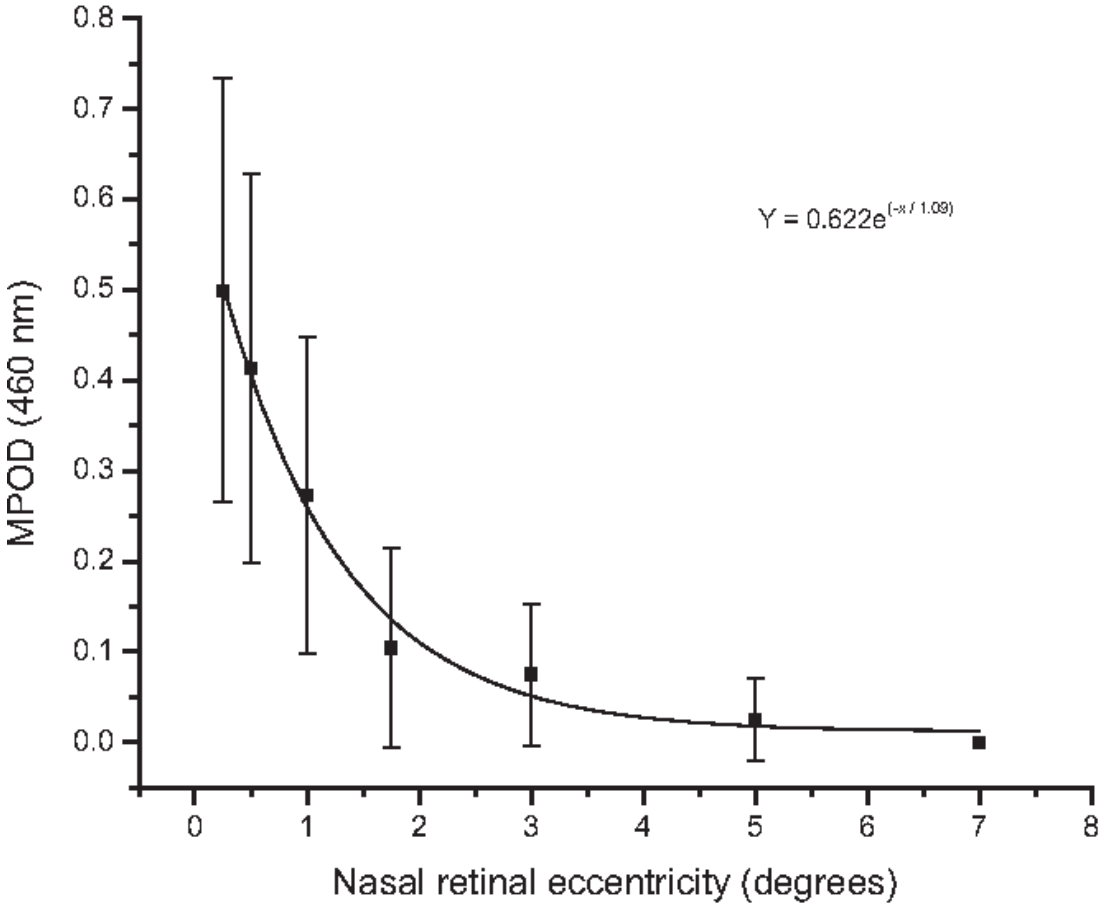


Figure 4.8: Mean spatial profile of MPOD (Adapted from Nolan et al,[342]).

More recently researchers have tried to identify other image-based methods which are less technically demanding and can measure MPOD objectively.

4.6.2 Image-based techniques

Non-invasive methods for assessment of carotenoid status have evolved considerably over the past few decades. Fundus autofluorescence has gained a lot of interest as the technique is objective and provides high-resolution and quantitative spatial distributions of MP. It is rapid and, apart from good fixation on a target, requires minimal patient cooperation. Fundus autofluorescence exploits the fluorescent properties of lipofuscin present and the predictable attenuation of the fluorescence by MP.[343] Lipofuscin is excited *in vivo* between 400 and 590 nm (peak excitation at 490-510 nm) and emits autofluorescence at 520-800 nm (peak emission at 590-630 nm).[344] At the fovea, excitation light within the absorbance range of MP is partially absorbed by the carotenoids, resulting in an area of reduced fluorescence. The Heidelberg Spectralis use the two-wavelength autofluorescence technique to measure MP and captures sets of images at two excitation wavelengths [226] (Figure 4.9).

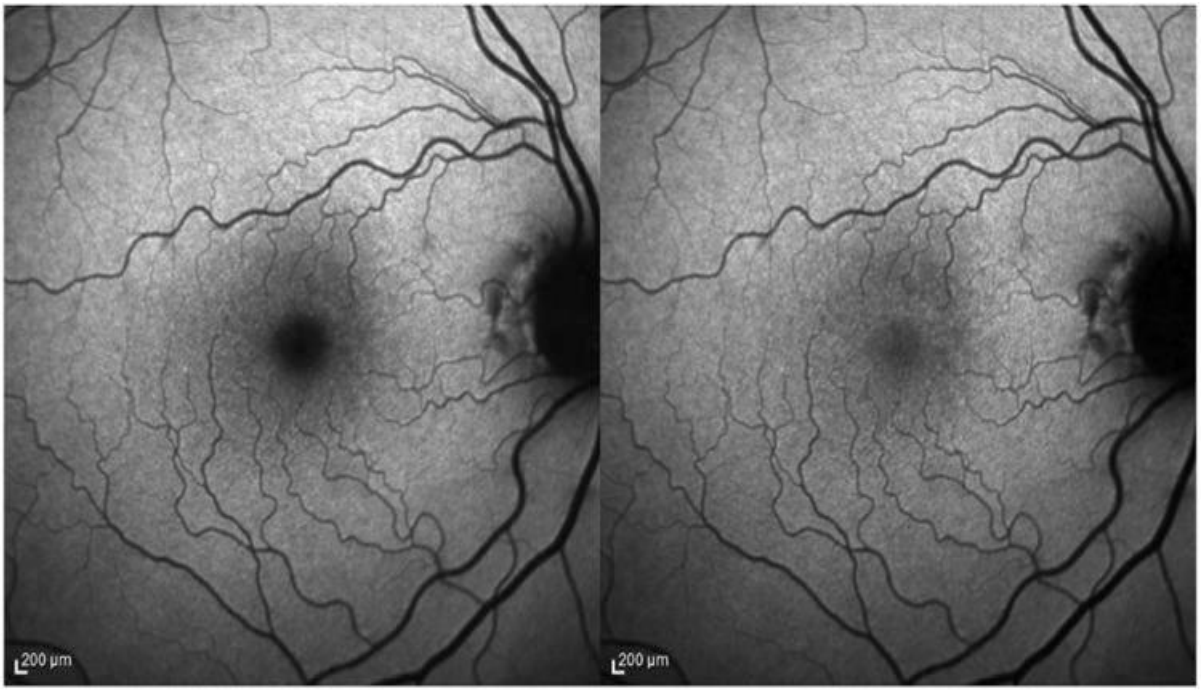


Figure 4.9: Image of macular pigment measured by Heidelberg Spectralis (Left, excitation wavelength at 488 nm; Right, excitation wavelength at 514 nm) (Adapted from Bernstein et al,[226]).

The Spectralis autofluorescence attenuation method is highly reproducible and provides a lot of spatial data.[345] The instrument calculates the average MPOD, standard deviation (SD) and range of MPOD levels along a series of concentric one-pixel width circles. The results are then plotted on a graph 0^0 to 15^0 with a red curve corresponding to the average MPOD at each eccentricity, a green curve corresponding to the SD of the average MPOD and a blue region corresponding to the high and low range of MPOD.[343] A reference point is typically taken at 9^0 eccentricity where the MPOD is defined as zero. Two other analysis eccentricities routinely used are 0.5^0 and 2^0 [343] (Figure 4.10).

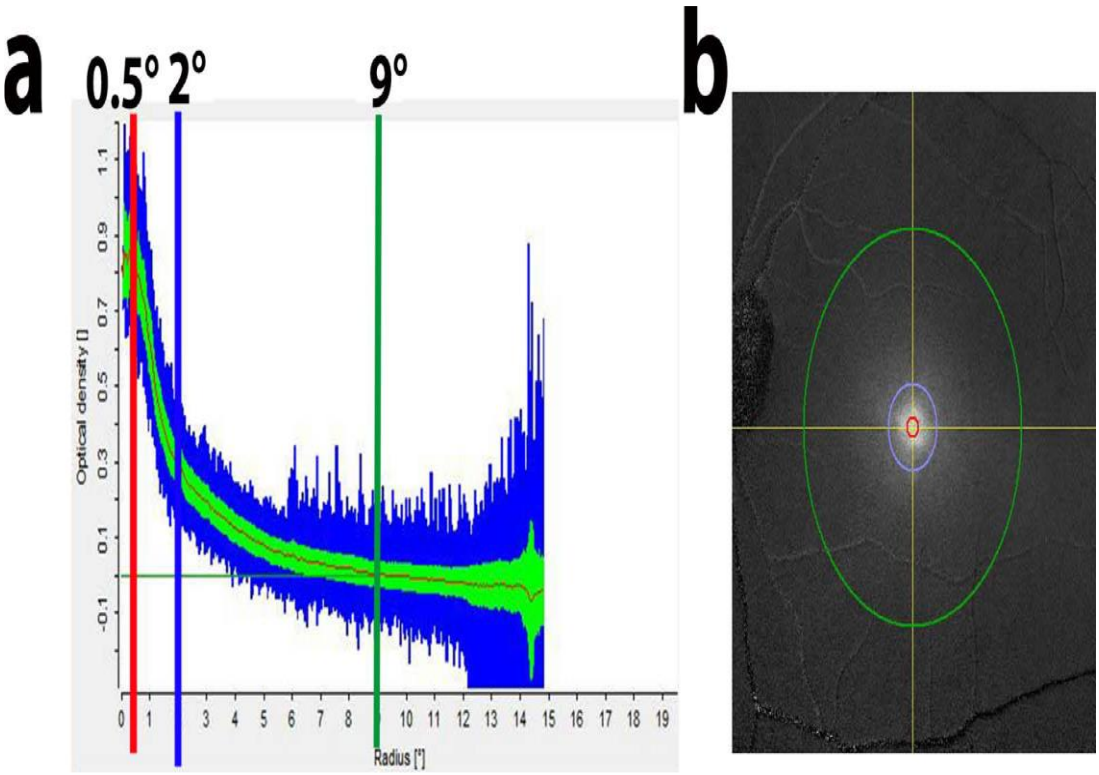


Figure 4.10: Macular pigment tracing from a healthy subject. (a) Macular pigment tracing at 0.5^0 (red), 2^0 (blue), and 9^0 (green) demarcated by the solid lines as indicated. (b) Macular pigment image showing the fovea and the degrees (0.5^0, red; 2^0, blue; 9^0, green) from the centre of the macula lutea (Adapted from Conrady et al, [343]).

A recent study, on subjects free of retinal disease, confirmed that measuring MP using autofluorescence imaging displays good concordance with c-HFP.[337] Furthermore, MP volume under the curve 9^0 gave the strongest correlations with lutein, zeaxanthin, lutein and zeaxanthin, which demonstrates the importance of capturing all of the carotenoid content of the macula rather than focusing on the MPOD at just one or two eccentricities.[343] Additional work, however, is needed to confirm its reliability across populations (e.g. patients with AMD and patients with diabetes). With the latest technology visually significant cataracts do not seem to suppress MP measurements

excessively especially when using MP under the curve 9^{0}.[343] Some drawbacks to the method, however, is the need for specialised equipment which is expensive, its bright light levels and the need for pupil dilation. Current generation autofluorescence techniques based on ocular imaging systems, however, look promising and may be well-suited for busy clinical practice settings in the near future.

4.6.2 Plasma analysis and dietary intake of carotenoids

Systemic measures of carotenoids are also used as a surrogate for ocular concentrations of these phytonutrients. Methods include HPLC analysis of plasma and dietary assessment. Plasma HPLC analysis of carotenoids have correlated well with assessment of ocular carotenoid status,[346-348] and are a good way of identifying patients who are adequately absorbing these phytonutrients. Plasma measurements, however, involve invasive blood draws and time-consuming analysis and are only available in a research setting.

Dietary assessment tools have also been used to assess carotenoid intake. Methods include food frequency questionnaires [FFQs], food diaries and 24-hour recalls. Owing to their relatively lower administrative costs and their ability to assess usual and longer-term intake, FFQs have historically been the method of choice for epidemiological studies.[349] Assessment of carotenoid intake in this way, however, has limitations. A common one is the overestimation of dietary intake as FFQs are greatly influenced by the accuracy of the portion size reported by the subject. Estimates obtained are also dependent on the use of accurate nutrient data sources. An issue with FFQs is that they may not always reflect food eaten by the population being examined. For example, FFQs developed for assessing lutein and zeaxanthin intake designed for an American population,[350] may not be valid for estimating these carotenoids in, for

example, an Irish population. Additionally, as lutein and zeaxanthin concentration in food varies with time and geographical origin, carotenoid databases used to analyse dietary intake need to be up to date and developed using foods from the country of origin of the population being studied. An accurate assessment is also difficult given that current databases generally report lutein and zeaxanthin as a combined value. When carotenoids are considered together it makes it even more difficult to determine their respective roles in eye health.[351]

A promising development in recent times is the 'lutein/zeaxanthin screener'. This new tool estimates carotenoid intake and offers advantages over other dietary assessment tools in that it is easy to use, quick, and cost-effective. Intake of lutein and zeaxanthin can be calculated (although reported as a combined value) as crude estimates (*i.e.* lutein/zeaxanthin score) based on dietary intake and relative bioavailability of four common xanthophyll containing food groups (eggs, broccoli, maize, and dark leafy vegetables).[352] The range of scores on the "lutein/zeaxanthin screener" is 0-75 and intake is categorised into low, medium and high. Dietary estimates of lutein and zeaxanthin combined have been shown to be positively and significantly correlated with plasma concentrations of these carotenoids ($r=0.329, p<0.001$), and this relationship remained after controlling for other variables such as age and body mass index (BMI).[352]

4.7 The need for alternative biomarkers

Despite some attempts, there is still no commercially available instrument that can successfully measure MPOD *in vivo* in clinical practice.[336] While the ''lutein/zeaxanthin screener' looks promising dietary intake assessed using FFQs is self-reported and only crudely quantified and as a result is subject to both bias and

error. In addition to, plasma analysis of carotenoids involves complex analysis. Consequently, there is merit in examining the predictive capacity of a range of more commonly measured proxy biomarkers; *i.e.* [clinical (blood pressure), plasma (lipoproteins, inflammatory markers) and anthropometric (abdominal fat)], which might be used to identify patients at risk of low MP. The capacity of more commonly-measured surrogate biomarkers to help identify people at risk of low MP (*i.e.* patients with Type 2 diabetes, older adults) will be explored in chapters seven and eight.

A REVIEW, EXPERIMENTAL WORK, RESULTS AND ANALYSIS

5. MACULAR PIGMENT IS LOWER IN TYPE 2 DIABETES COMPARED WITH TYPE 1 DIABETES AND NORMAL CONTROLS.

5.1 Abstract

Purpose

This cross-sectional study was designed to investigate the optical density of MP in Type 1 and Type 2 diabetes subjects relative to normal controls.

Methods

One hundred and fifty subjects were recruited to the study and divided into one of the three study groups based on their health status, as follows: Group 1: Healthy controls; Group 2: Type 1 diabetes; Group 3: Type 2 diabetes. Macular pigment optical density at 0.5° of retinal eccentricity was measured using c-HFP. Dietary intake of macular carotenoids was quantified using a lutein and zeaxanthin FFQ. Diabetes type, duration, medication, smoking habits, HbA1c, and plasma lipid levels were recorded, whereas VA, BMI, and diabetic retinopathy grade were measured for each participant.

Results

One-way analysis of variance revealed a statistically significant difference in BMI, age, HDL cholesterol and HbA1c between the three groups ($p<0.01$ for all). Chi-square analysis revealed a statistically significant difference in diabetic retinopathy distribution ($p<0.01$). None of these variables exhibited a statistically significant correlation with MPOD for any study group ($p>0.05$ for all). There was no difference in dietary carotenoid intake between groups. Macular pigment optical density was lower among Type 2 diabetes subjects (0.33 ± 0.21) compared with Type 1 diabetes (0.49 ± 0.23) and normal controls (0.48 ± 0.35). General linear model analysis,

including age, BMI, diabetes duration, diabetic retinopathy status, HDL, and HbA1c as covariates, revealed a statistically significant effect of diabetes type on MPOD ($F = 2.62$; $p=0.04$).

Conclusion

Macular pigment optical density was statistically significantly lower in Type 2 diabetes compared with Type 1 diabetes and normal controls. Although BMI was higher in the Type 2 diabetes group, the lower MPOD levels observed among Type 2 diabetes seem not to be attributable to differences in the dietary carotenoid intake or the specific presence of diabetes, diabetic control, duration, or diabetic retinopathy.

5.2 Introduction

Neurodegenerative diseases of the retina such as AMD and diabetic retinopathy are leading causes of worldwide blindness.[353] Although the relationship between AMD and MPOD has been widely reported,[221, 339] only a small number of studies have focused on MPOD and carotenoid intake in diabetes mellitus,[6, 15] a condition similarly known to cause oxidative damage in the retina.[354]

Diabetes, a lifelong progressive disease, is the result of the body's inability to produce insulin or use insulin to its full potential and is characterised by high circulating glucose.[355] Diabetic retinopathy represents the most common diabetic eye disease, and there is strong evidence that oxidative stress plays an important role in its development.[354, 355] Chronic hyperglycaemia causes oxidative stress,[355] which results in microvascular complications at the retina, where the neuronal elements responsible for vision are located. The relationship between hyperglycaemia, changes in redox homeostasis, and oxidative stress are key events in the pathogenesis of diabetic retinopathy.[355] Oxidative stress is also involved in the initiation and progression of obesity and diabetes mellitus.[356] The links between Type 2 diabetes and obesity are firmly established.[357] Type 2 diabetes now also affects a much younger population because of sedentary lifestyles and increases in calorific intake and accounts for more than 90% of all cases of diabetes.[358] The development of ocular complications in diabetes is related to disease control and longevity.[11, 359, 360] After 20 years with diabetes, 75% of patients will have some form of diabetic retinopathy.[360] Apart from good systemic control of blood sugar levels, hypertension, lipid profiles, and renal function, current treatment modalities for diabetic retinopathy are limited to laser photocoagulation [11] and/or intravitreal injections.[12] These are effective modes of

treatment but they also have their limitations and side effects. New modalities should, therefore, be preventative in nature and ideally implemented long before overt clinical symptoms develop. Macular pigment is believed to possess antioxidant properties and to limit retinal oxidative damage by absorbing harmful short-wavelength blue light.[3] Macular pigment is highly concentrated at the central macula and is composed of the dietary hydroxyl-carotenoids lutein, zeaxanthin, and *meso*-zeaxanthin.[46] These hydroxyl carotenoids are found in the retina to the exclusion of all other 700 carotenoids found in nature.[312] Concentrations of carotenoids in human plasma and deposition of the macular carotenoids in the retina to form MP are highly variable and reflect not only dietary intake but also factors such as carotenoid chemistry,[361] individual efficacy of absorption,[70] fat intake,[16] competition among carotenoids for absorption,[17] cholesterol and lipoprotein status,[18, 71] metabolic status,[6] body composition, and BMI.[17, 54] The relationship between MP and diabetes is only now attracting research interest, possibly as a result of the outcomes of clinical trials, which demonstrate a protective effect of lutein and zeaxanthin supplementation in another oxidative stress-related condition, AMD.[221, 339] One experimental study on diabetic rats demonstrated a reduction in retinal oxidative damage after carotenoid supplementation.[14] Lutein and zeaxanthin intake has also been shown to improve macular oedema in diabetic retinopathy patients.[15] These results suggest that MP supplementation has the potential to inhibit or delay the development of macular disease in patients with diabetes.

This study was designed to investigate the cross-sectional relationship, if any, between diabetes and central MPOD and to explore the influence of potential explanatory factors, including HbA1c, plasma lipid levels, BMI, and dietary carotenoid intake on

any relationship that might be observed.

5.3 Materials and Methods

Subjects

Diabetic participants and normal control subjects were recruited for this cross-sectional, case-control study at the Mater Misericordiae University Hospital in Dublin, Ireland. The study was approved by the Institutional Research Ethics Committee and adhered to the tenets of the Declaration of Helsinki. Informed consent was obtained from all subjects before enrolment and examination. Generic inclusion criteria were as follows: subjects were to be older than 18 years, not having taken dietary supplements containing lutein, zeaxanthin or *meso*-zeaxanthin over the 6-month period before the study, and logMAR VA better than 0.2 (6/9) in the study eye. For normal (control) subjects, exclusion criteria included any sign of retinal or ocular abnormality and the presence of Type 1 or Type 2 diabetes. Patients with diabetes were excluded if they exhibited signs of ocular comorbidity (e.g. glaucoma, cataract, AMD), had previously undergone any form of treatment for diabetic retinopathy or maculopathy, or if they exhibited any signs of proliferative retinopathy or maculopathy. The study eye was selected using the eye with better VA, or, in cases of equal acuity, the right eye was selected as standard. Subjects were assigned into one of three study groups based on their ocular health status and diabetes type as follows: Group 1: Non-diabetic controls; Group 2: Type 1 diabetes; Group 3: Type 2 diabetes.

Demographic and lifestyle information

Demographic information, including age, gender, race, history of smoking (current smoker, ex-smoker, and never smoked) and duration of smoking in years was collected in relation to each subject.

Diabetes classification and diabetic retinopathy grading

The following diabetes-related information was recorded for each diabetes participant: diabetes duration, diabetes type, and diabetic retinopathy grade. Subjects were classified based on their ocular health status and diabetes type as non-diabetic controls, Type 1 diabetes, and Type 2 diabetes. The duration of disease was recorded in years. Diabetic retinopathy was graded according to modified 2-field early treatment diabetic retinopathy study (EDTRS) protocol-grade range R0 M0 to R2 M0 by consultant ophthalmic surgeon, Mr Paul Connell, Mater Private Hospital. Medications for diabetes including insulin and oral hypoglycaemics were noted. Glycated haemoglobin (%) was also recorded.

Anthropometric measurement

Anthropometric measurements including weight and height were obtained using standardised techniques. Body weight was measured to the nearest 0.2 kg using a Seca Compact Digital Floor Scale 111, model 888 (Seca Limited), whereas, height was measured to the nearest 0.5 cm using a collapsible "Leicester Height Measure" stadiometer (CMS Weighing Equipment). Body mass index was given as weight (in kilograms)/height (in square metres, (kg/m^2)).

Plasma analysis

Plasma blood levels including total cholesterol (TC) in millimoles per litre (mmol/L), LDL (mmol/L), HDL (mmol/L), TGs (mmol/L) and HbA1c (%) were recorded for each subject.

Lutein and zeaxanthin intake.

Dietary carotenoid intake was estimated using the lutein/zeaxanthin questionnaire

(Carotenoid & Health Laboratory, Jean Mayer USDA Human Nutrition Centre on Aging, Tufts University, USA).[350] Lutein, zeaxanthin and total lutein and zeaxanthin in micrograms (mg) per day were quantified.

Macular pigment optical density measurement

Macular pigment absorbs blue light and is optically undetectable at 6° to 8° eccentricity.[44] In this study, MPOD was determined using the Macular Metrics Clinical Densitometer (Macular Metrics, Rehoboth, MA), which is based on the principle of HFP.[334] The basic measurement procedure involves presenting a small test stimulus that alternates between a measuring wavelength (458 nm), which is absorbed by MP and a reference wavelength (540 nm) not absorbed by the pigment. The subject adjusts the intensity of the measuring wavelength (458 nm) luminance until there is minimum flicker (matching luminance). The luminance of the reference wavelength (540 nm) remains constant. The ratio of the amount of measuring wavelength (458 nm) light required to achieve the endpoint of matching luminance, or minimum flicker, for foveal and parafoveal readings is a measure of the amount of pigment present, and the logarithm of this ratio represents the optical density of MP. Before using the densitometer, all subjects were shown an explanatory video describing the method for recording null flicker matches. The measurement was conducted by a single examiner who was highly skilled and experienced with the technique. All subjects were naive to the HFP test. The MPOD measurement comprised the average of six readings (computed as the radiance value at which the subject reported null flicker) taken centrally at 0.5° retinal eccentricity and again at 7° retinal eccentricity. The target used for measuring MPOD at 0.5° eccentricity was a solid disc of 0.5° arc radius, and the parafoveal measurement was taken by asking the subject to fixate on a red light located precisely at 7° from central fixation. Measurements were deemed reliable and

acceptable only when the SD of null flicker responses was below 0.1. A c-HFP procedure was adopted, whereby flicker frequency was optimised for each participant before the measurement of MPOD. The starting flicker was set at 10 Hz to 11 Hz. If the subject was unable to find no/minimum flicker, this frequency was increased until no/minimal flicker could be identified. If the null one was too wide, the flicker frequency was reduced. The flicker frequency was adjusted until the subject found a narrow null zone.

Statistical analysis

The statistical software package SPSS for Microsoft Windows (v.21.0; IBM Corp., Armonk, NY) was used for analysis. Data are presented as mean ± standard deviation throughout. The data were tested for normality using the Kolmogorov–Smirnov test. One-way analysis of variance (ANOVA) was used to test for differences in normally distributed study parameters between groups, whereas the Kruskal–Wallis test was used to test for differences in group medians in non-normally distributed data. The Chi-square test was used to compare categorical data across the three study groups. Pearson's product-moment correlation tests were performed to assess the relationship between MPOD and other study variables where appropriate. A general linear model approach was used to explore the relationship between diabetes type, other potential explanatory variables, and the dependent variable, MPOD. The level of statistical significance was set at the standard $p<0.05$.

5.4 Results

One hundred and fifty subjects were recruited to the study and divided into 1 of the 3 study groups on the basis of their ocular health status and diabetes type, as follows: Group 1: Non-diabetic controls (n = 48; male = 20, female = 28); Group 2: Type 1

diabetes (n = 34; male = 17, female = 17); Group 3: Type 2 diabetes (n = 68; male = 43, female = 25). Kolmogorov–Smirnov analysis revealed that BMI, TC, lutein intake, zeaxanthin intake, and MPOD data were normally distributed (p>0.05 for each), whereas all remaining variables exhibited non-normal distributions. Accordingly, parametric tests were applied, where relevant, to normally distributed data, and nonparametric equivalent tests to non-normally distributed data. A comparison of the demographic and clinical characteristics of each group is presented in Table 5.1.

Table 5.1: Demographic and clinical findings according to classification group.

Variable	Non-diabetic controls	Type 1 Diabetes	Type 2 Diabetes	*p* value
Sex	Male = 20; Female =28	Male = 17; Female = 17	Male = 43; Female = 25	<0.07*
Age (years)	52.48 ± 16.03	43.67 ± 12.98	62.67 ± 11.31	**<0.01**†
BMI (kg/m^2)	27.7 ± 4.8	27.33 ± 4.77	31.44 ± 6.16	**<0.01**‡
Smoking duration (years)	2.13 ± 0.62	2.35 ± 0.60	2.25 ± 0.65	0.33†
Diabetes mellitus duration (years)	-	22.73 ± 10.85	9.54 ± 7.33	**<0.01**†
HbA1c (%)	-	7.9 ± 1.08	6.88 ± 1.09	**<0.01**†
TG (mmol/L)	-	1.37 ± 2.06	1.55 ± 0.85	0.55†
HDL (mmol/L)	-	1.52 ± 0.46	1.14 ± 0.28	**<0.01**†
LDL (mmol/L)	-	2.37 ± 0.70	2.20 ± 0.74	0.15†
TC (mmol/L)	-	4.31 ± 0.82	4.03 ± 0.89	0.14‡
MPOD (SD)	0.48 ± 0.35	0.49 ± 0.23	0.33 ± 0.21	**<0.01**‡
Lutein Intake (mg/day)	0.95 ± 0.21	1.06 ± 1.29	0.95 ± 1.73	0.93‡
Zeaxanthin Intake (mg/day)	0.11 ± 0.12	0.16 ± 0.26	0.12 ± 0.25	0.54‡
Total Lutein & Zeaxanthin Intake, (mg/day)	1.07 ± 1.25	1.23 ± 1.34	1.07 ± 1.83	0.88‡
Non-Proliferative Diabetic Retinopathy	-	Yes = 23 (68%) No = 11 (32%)	Yes = 24 (35%) No = 44 (65%)	**<0.01***

***** *Chi-square test* † *Kruskal-Wallis test* ‡ *One-way Analysis of Variance.*

Abbreviations: ANOVA, one way analysis of variance; BMI, body mass index; kg/m^2, kilogrammes per metre squared; HbA1c, glycated haemoglobin level; HDL, high density lipoprotein; LDL, low density lipoprotein; mg/day; micrograms per day; mmol/L, millimoles per litre; TC, total cholesterol; TG, triglycerides.

Post hoc analysis (Tukey test) revealed that MPOD was statistically significantly lower in Type 2 diabetes compared with both normal controls (p=0.02) and Type 1 diabetes (p=0.03), whereas the difference in MPOD between Type 1 diabetes and normal controls was not statistically significant (p=0.99). The distribution of MPOD for each of the study groups is presented in Figure 5.1

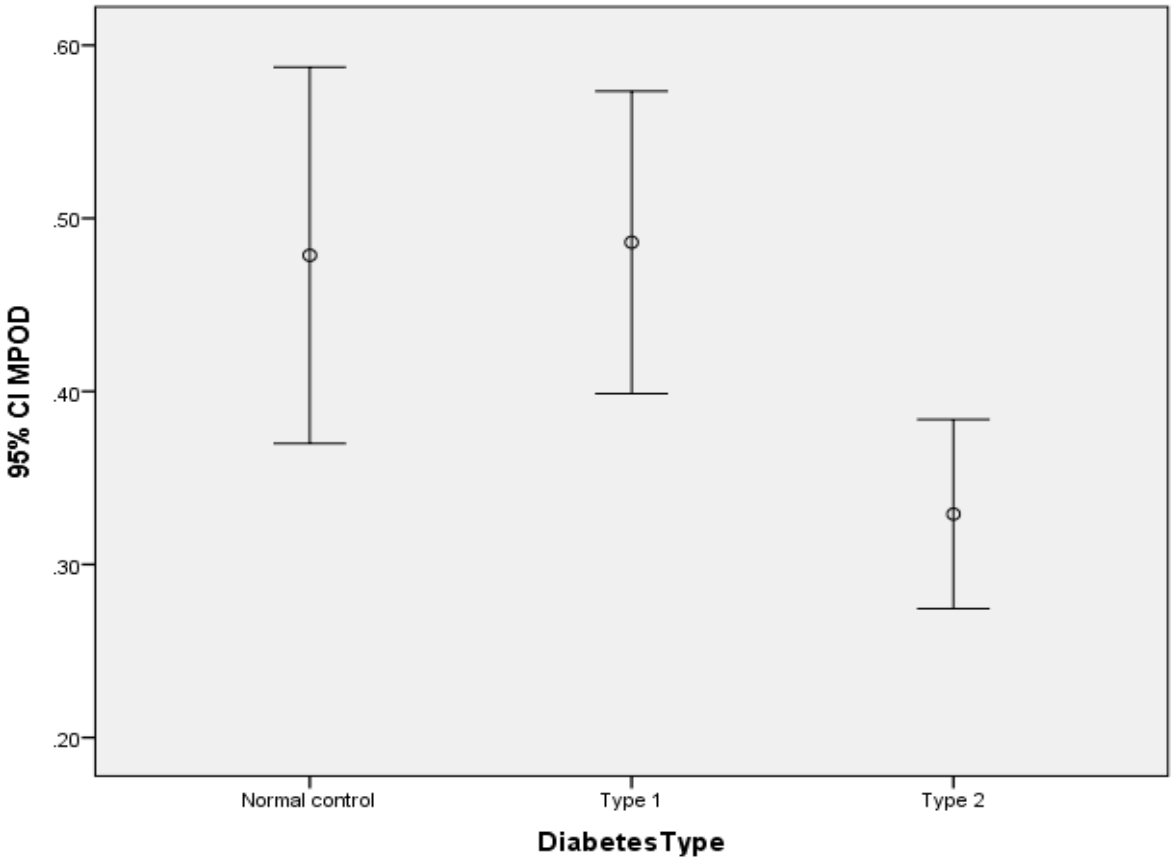


Figure 5.1: MPOD distribution (mean ± 95% confidence intervals) according to diabetes status, illustrating the substantially lower MPOD levels among Type 2 diabetes subjects.

A general linear model analysis confirmed a significant effect of diabetes type on the dependent variable, MPOD (p=0.04), but no effect of potentially confounding

variables including age, BMI, diabetic retinopathy status, HDL cholesterol, or HbA1c on MPOD (p=0.16 – 0.74). While there was a significant difference in age between the three groups; Type 1 (mean age = 43.67±12.98); Type 2 (mean age = 62.67±11.31) and non-diabetic controls (mean age = 52.48±16.03) (p<0.01); age was controlled for in the general linear model and had no effect on MPOD (p=0.128). In addition, age, including controlling for confounding, was not significantly correlated with MPOD (r=-0.175; p=0.138). The distribution of MPOD according to diabetic retinopathy status, which demonstrated no influence on MPOD in the general linear model analysis (p=0.45), is presented in Figure 5.2.

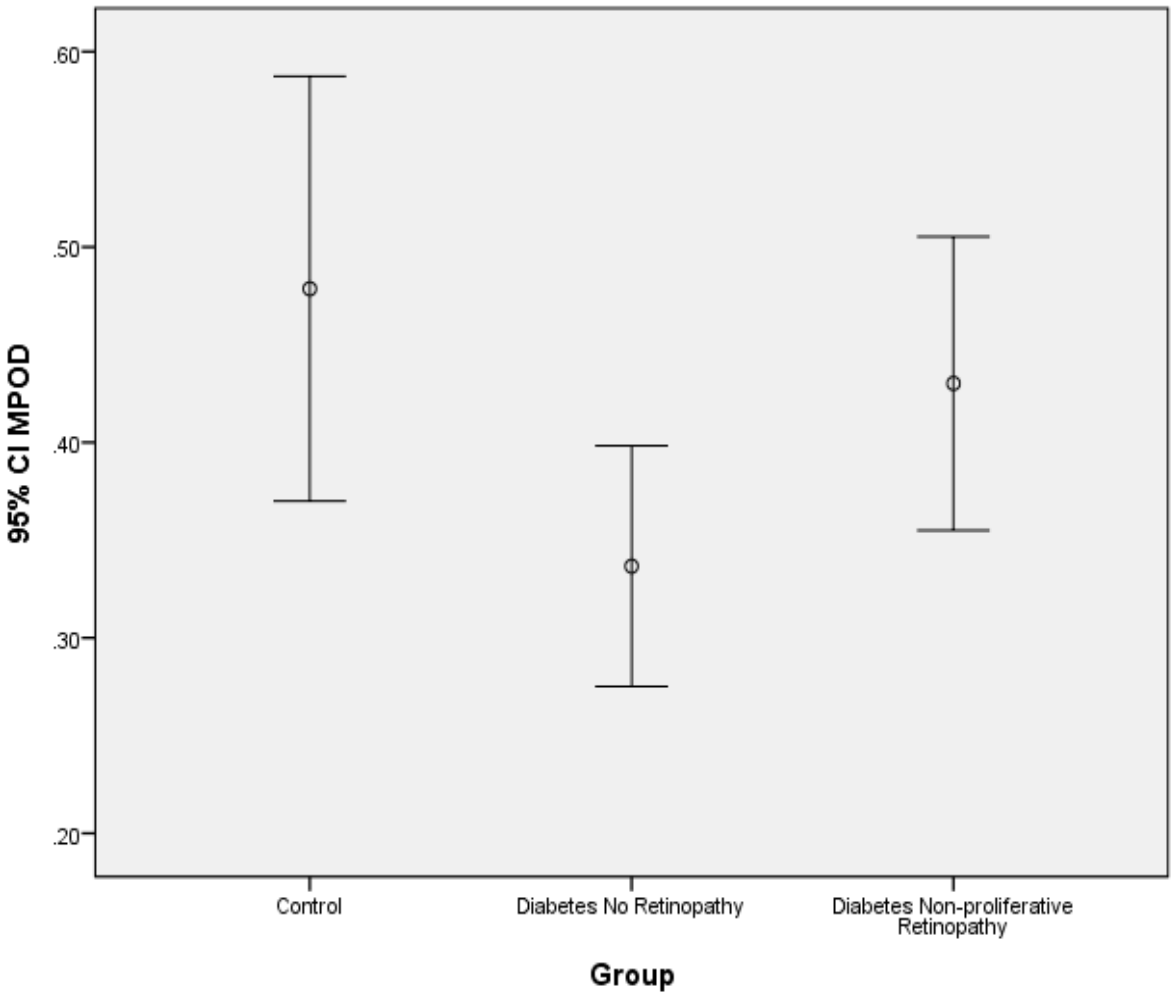


Figure 5.2: The distribution of MPOD (mean ± 95% confidence intervals) according

to diabetic retinopathy status, illustrating the significant overlap between groups.

Body mass index was statistically significantly higher in Type 2 diabetes compared with normal controls ($p<0.01$) and Type 1 diabetes ($p<0.01$), whereas the difference in BMI between Type 1 diabetes and normal controls was not statistically significant ($p=0.75$). Body mass index, however, including controlling for confounding was not statistically significantly correlated with MPOD (r=0.08, $p=0.51$). The distribution of BMI for each of the study groups is presented in Figure 5.3.

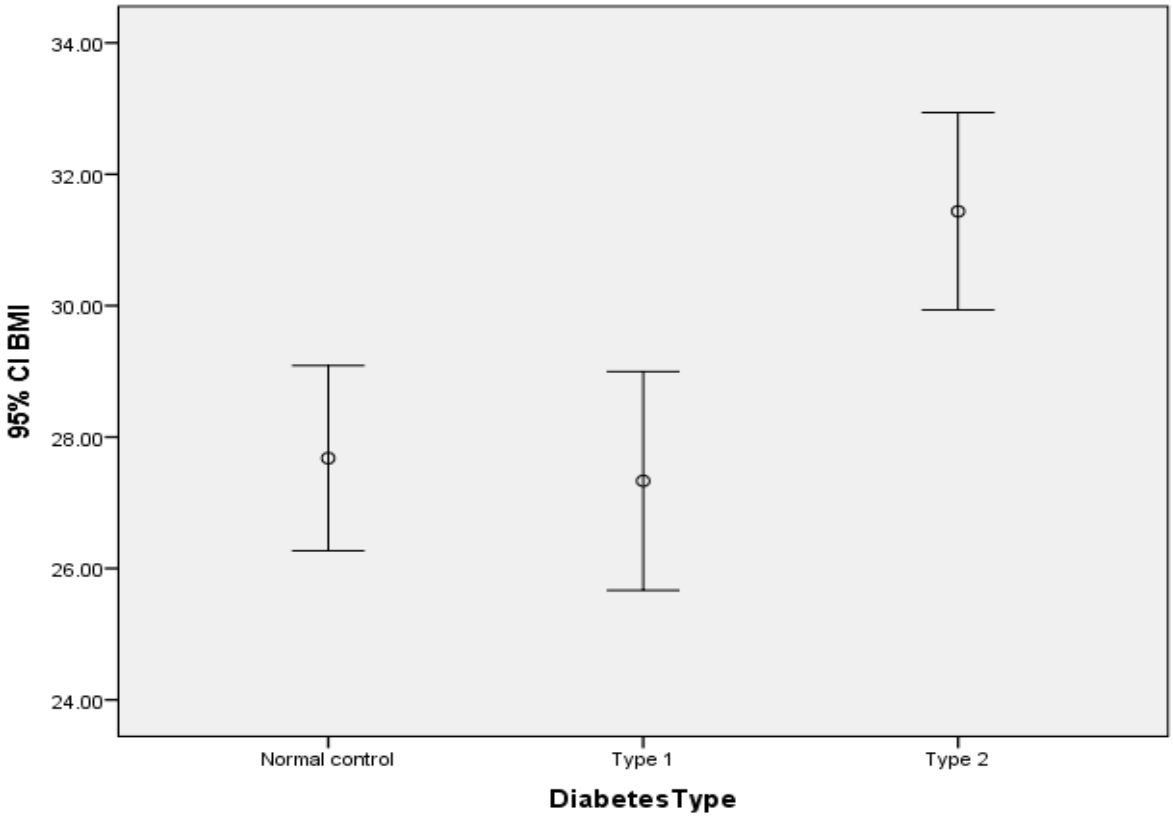


Figure 5.3: Body mass index distribution (mean ± 95% confidence intervals) according to diabetes status, illustrating substantially higher BMI levels among Type 2 diabetes subjects.

5.5 Discussion

This study demonstrates that MPOD levels are significantly lower in Type 2 diabetes when compared with Type 1 diabetes, which represents an entirely novel finding. This observation is significant given that similar dietary carotenoid intake was observed among the study groups and in the context of shorter diabetes duration among the Type 2 diabetes subjects. Similarly, the presence of non-proliferative diabetic retinopathy seems not to influence MPOD levels, which are, surprisingly, slightly higher on average in those with retinopathy compared with those without retinopathy, although lower in both diabetic groups compared with normal controls. The study further demonstrates that the MPOD levels in Type 2 diabetes are significantly lower than in normal controls, a finding, which is in general agreement with those of previous investigations that have explored the relationship between MPOD and diabetes. It has been shown, for example, that Type 2 diabetes subjects had lower MPOD when compared with non-diabetic controls, while Type 2 diabetes with mild non-proliferative retinopathy exhibited similar MPOD levels to Type 2 diabetes without retinopathy.[6] In addition, plasma lutein and zeaxanthin concentrations, which have been shown to be positively and significantly related to MPOD in normal subjects,[49] have been shown to be significantly lower in diabetes compared with normal subjects.[15] In more advanced cases of diabetic eye disease, subjects with Grade 2 maculopathy were found to have significantly lower MPOD than those without maculopathy.[9] The apparent relationship between low MP levels and increasing severity of maculopathy prompted those study investigators to implicate oxidative stress as a causative factor, a concept that merits further discussion here.[9]

Macular pigment optical density has a slow biological turnover, as it reflects the local balance between pro-oxidant stresses and antioxidant defences in the retina.[49] The retina is particularly susceptible to oxidative stress because of its high oxygen consumption, the high proportion of PUFAs, and exposure to visible and short-wavelength blue light.[291] There is also considerable evidence that hyperglycaemia results in the generation of ROS, ultimately leading to increased oxidative stress in the retina,[354, 355] which may play an important role in the aetiology of diabetic complications. Poor metabolic control and longer duration of diabetes are directly linked to the prevalence of diabetic retinopathy,[359] whereas oxidative stress can be significantly reduced with aggressive control of hyperglycaemia.[362] We found no association, however, between MPOD and either HbA1c or diabetes duration in this study. Paradoxically, despite their lower MPOD, metabolic control appeared better, diabetes duration was shorter, and diabetic retinopathy prevalence was lower for Type 2 compared with Type 1 diabetes subjects. Although HbA1c has previously been shown to be inversely and significantly related to MPOD among a smaller sample of Type 2 diabetes subjects,[6] our findings imply that lower MPOD in Type 2 diabetes is not attributable to the diabetic condition, duration, or level of control, and suggests a need to explore alternative explanatory causes. Furthermore, while there was a significant difference in age between the three groups ($p<0.01$), and some studies have demonstrated a decline in MPOD with age, [49, 55, 56] although not all [57, 58] age was not a predictor of MPOD on general linear model analysis. Also, we found no correlation between MPOD and age, while controlling for all other variables.

Although BMI measures do not provide a precise indicator of adiposity, it is likely that the statistically significantly higher BMI levels observed in the Type 2 diabetes

group here, which averaged above World Health Organisation (WHO) defined BMI levels indicative of obesity (BMI>30),[363] are reflective of excess adiposity in this group compared with the normal control and Type 1 diabetes groups. To date, there has been very little consensus on the relationship between types of obesity and MPOD. One study reported a lack of an association between MPOD and various obesity indices, including WC, WHpR, and BMI, in a South-Indian population.[364] Conversely, other studies have shown significant inverse relationships between body fat and MPOD in humans,[16, 17, 54] however, these relationships differed between men [16] and women.[17] Lutein and zeaxanthin are known to accumulate in adipose tissue,[257] and lower MPOD values have also been reported among individuals exhibiting body fat measuring greater than 27% when compared with those with lower body fat.[54] Higher body fat percentage even within relatively healthy limits is associated with lower tissue lutein and zeaxanthin status [53] and renders the macular carotenoids less available to retinal tissue.[68] Distribution of body fat in the body is also important, and it has been shown that lutein/zeaxanthin concentrations in adipose tissue differ according to body site, with levels demonstrably higher in abdominal fat than in the gluteofemoral fat depot.[67] Interestingly, abdominal obesity is now considered an important risk factor and predictive indicator for the development of Type 2 diabetes and cardiovascular disease.[365] Waist-to-height ratio has more recently been shown to have superior discriminatory power for detecting cardiovascular risk factors in both sexes [366] and should be considered as a screening tool for diabetes, hypertension, and cardiovascular disease in men and women.[366] Future studies investigating the link between MPOD and obesity should, therefore, encompass more refined methods of body fat assessment including bioelectrical impedance analysis and WHtR,[366] because BMI measurements alone may not be adequate to provide a true indication of adiposity, and this could

explain the lack of an association between MPOD and BMI in our study. Diets, which are high in fat and low in carotenoids, have been linked with increased oxidative stress.[367] Obesity is also independently associated with increased oxidative stress [69, 367, 368] and an increased BMI has also been shown to be associated with increased risk of DNA damage due to oxidative stress.[369] Plasma 8-OHdG, for example, a known sensitive marker of oxidative DNA damage and total systemic oxidative stress *in vivo*, has been shown to be positively correlated with BMI in people with Type 2 diabetes mellitus.[370] Adipose tissue produces bioactive substances called adipokines, which induce the production of ROS by a combination of mechanisms including mitochondrial and peroxisomal oxidation of fatty acids and overconsumption of oxygen,[69, 368] thereby, initiate a process of oxidative stress.[69, 368] Recent studies have highlighted the role of increased abdominal fat mass as a key driver of inflammation in Type 2 diabetes,[371] a state closely associated with increased oxidative stress,[69, 368] and macrovascular disease.[371] Chronic inflammation induces changes in metabolic pathways and is believed to play a significant role in the progression from obesity to Type 2 diabetes.[372, 373] Despite the lack of any direct association between BMI and MPOD observed herein, it is plausible to suggest that; 1) more refined methods of body fat assessment such as bioelectrical impedance analysis [374] and WHtR [366] might reveal an association that could explain the lower MPOD values observed in Type 2 diabetes subjects here and; 2) that the combined effect of increased competition for lutein/zeaxanthin deposition and increased inflammation and oxidative stress levels in association with higher BMI/body fat might explain, at least in part, the lower MPOD levels observed in the Type 2 diabetes group. There is no significant difference in MPOD between patients with or without retinopathy indicating that retinopathy status seems not to be the main driver in influencing MP levels.

High-density lipoprotein differences observed between Type 1 and Type 2 diabetes could also contribute to the lower MP levels observed in the Type 2 diabetes group. Macular pigment carotenoids are primarily transported by HDL in plasma.[18, 71] It has been suggested that mechanisms governing the retinal capture and/or stabilisation of these carotenoids in the retina may be subject to HDL influence, by affecting receptor-mediated uptake of these carotenoids from plasma.[71] High-density lipoprotein deficiencies are associated with lutein and zeaxanthin tissue deficiencies, most notably in the retina.[18] Furthermore, it has been suggested that individuals exhibiting elevated plasma TG concentrations and concurrently reduced plasma HDL concentrations may have a related and reduced capacity to transport lutein in plasma.[71] Such features are very characteristic of Type 2 but not Type 1 diabetes.[375] Lipoprotein profile is adversely affected by insulin resistance and might mechanistically explain (by eliciting a reduced capacity to transport macular carotenoids), why Type 2 diabetes is associated with lower MP levels compared with Type 1 diabetes where insulin resistance is much less prominent. A more comprehensive investigation into the association between lipoprotein profile, MPOD, and dietary carotenoid intake among diabetes subjects is therefore warranted. Average macular carotenoid intake was similar for the three study groups (p=0.88) and at levels that are consistent with the lower limits of average daily carotenoid intake, (ranges from 1.1 to 1.6 mg/day).[49] Although dietary intake is the primary driver of tissue carotenoid levels,[3] the relationship between diet and deposition of these pigments in the retina is moderated by a number of factors. Retinal capture and/or stabilisation of these carotenoids in the macula, may, for example, be subject to influence by obesity-induced inflammation and oxidative stress,[372, 373] competition between adipose and retinal tissue for the dietary carotenoids,[17, 53, 54] impaired transport of circulating lutein/zeaxanthin,[18, 71]

and/or genetic influence,[224] which may help to explain the significant effect of Type 2 diabetes on MPOD.

5.6 Limitations

Limitations to this cross-sectional case-control study include the use of a dietary questionnaire designed for an American population among European participants, lack of plasma carotenoid analysis, lack of detailed body fat analysis, and the absence of other clinical parameters such as inflammatory markers. Further research is needed in the diabetic population to uncover the relationship between disease progression, BMI including WHtR, body fat composition, plasma cholesterol, inflammatory status, and dietary carotenoid intake, as well as the mechanism of retinal damage in the presence of low MPOD.

5.7 Conclusion

The effects of the adiposity, insulin resistance, inflammation, and HDL suppression, characteristics that differentiate Type 2 from Type 1 diabetes, on MPOD should be particularly emphasised. A longitudinal study, comparing MPOD in Type 1 and Type 2 diabetes with parameters such as lipoprotein profile (including LDL, HDL, and TGs),[18, 71] obesity indices (including WHtR, bioelectrical impedance analysis, BMI, WC, and waist-to-hip ratio (WHpR) measurements),[366] and dietary intake of lutein and zeaxanthin, [350] would certainly provide a more holistic understanding of the relationship between MPOD and BMI in Type 2 diabetes patients over time. Certain features such as obesity,[356] low HDL, and raised TGs are characteristic of Type 2 but not Type 1 diabetes,[375] and these characteristics may indeed affect the transport, uptake, and stabilisation of these carotenoids in the retina and would, therefore,

warrant further investigation.

6. A REVIEW OF THE PUTATIVE CAUSAL MECHANISMS ASSOCIATED WITH LOWER MACULAR PIGMENT IN DIABETES MELLITUS.

6.1 Abstract

Purpose

Macular Pigment confers potent antioxidant and anti-inflammatory effects at the macula, and may therefore, protect retinal tissue from the oxidative stress and inflammation associated with ocular disease and aging. There is a body of evidence implicating oxidative damage and inflammation as underlying pathological processes in diabetic retinopathy, a major cause of vision impairment and blindness. Macular pigment has therefore become a focus of research in diabetes. This review explores the currently available evidence pertaining to MP levels in diabetes, and illuminates the potential metabolic perturbations implicated in MP depletion in diabetic eye disease.

Methods

This review was carried out in two stages. Firstly we identified all relevant published articles from human and animal studies which reported on the relationship between MP (lutein and/or zeaxanthin and/or *meso*-zeaxanthin) and diabetes (Type 1 & Type 2), up until the year 2019. The second part of the search involved identifying publications which investigated the relationship between the metabolic perturbations typically associated with diabetes, and Type 2 diabetes in particular (e.g. adiposity/dyslipidaemia) and MP. PubMed, Google Scholar, Mendeley, Scopus,

Cochrane Library and the ISRCTN registry were used to search for literature of relevance to MP and diabetes. Relevant citations in the literature located through our search were also appraised.

Results

Metabolic co-morbidities commonly associated with Type 2 diabetes such as overweight/obesity, dyslipidaemia, hyperglycaemia and insulin resistance, may have added and independent relationships with MP. Increased adiposity and dyslipidaemia may adversely affect MP by compromising the availability, transport, and assimilation of these dietary carotenoids in the retina. Furthermore, carotenoid intake may be compromised by the dietary deficiencies characteristic of Type 2 diabetes, thereby, further compromising redox homeostasis.

Conclusion

Candidate causal mechanisms to explain the lower MP levels reported in diabetes include increased oxidative stress, inflammation, hyperglycaemia, insulin resistance, overweight/obesity and dyslipidaemia; factors, which may negatively affect redox status, and the availability, transport and stabilisation of carotenoids in the retina. Further study in a diabetic population is warranted to fully elucidate these relationships.

6.2 Introduction

The macula is an oval shaped area at the centre of the retina which consists of a dense collection of light-sensitive cone cells responsible for central, high-resolution vision and colour perception.[376] At the centre of the macula lies the fovea, where MP is located. Macular pigment is comprised of three carotenoids; lutein, zeaxanthin and the retinal metabolite of lutein, *meso*-zeaxanthin.[46,226] Lutein and zeaxanthin are not synthesised *de novo* in humans but are exclusively of dietary origin, typically derived from a diet rich in coloured fruit and vegetables,[22] while *meso*-zeaxanthin can be generated by conversion from retinal lutein, [219] or may be obtained from dietary sources such as trout and salmon.[220] The highest concentration of MP is found in the receptor axon layer of the foveola, while in the parafovea, MP is located in the inner plexiform layers,[44,43] an area of the retina which is primed for the generation of ROS and consequently, oxidative damage.[291] These hydroxyl-carotenoids are selectively located in the macula to the exclusion of all other dietary carotenoids. Figure 6.1 highlights the location of MP and the macula within the eye.

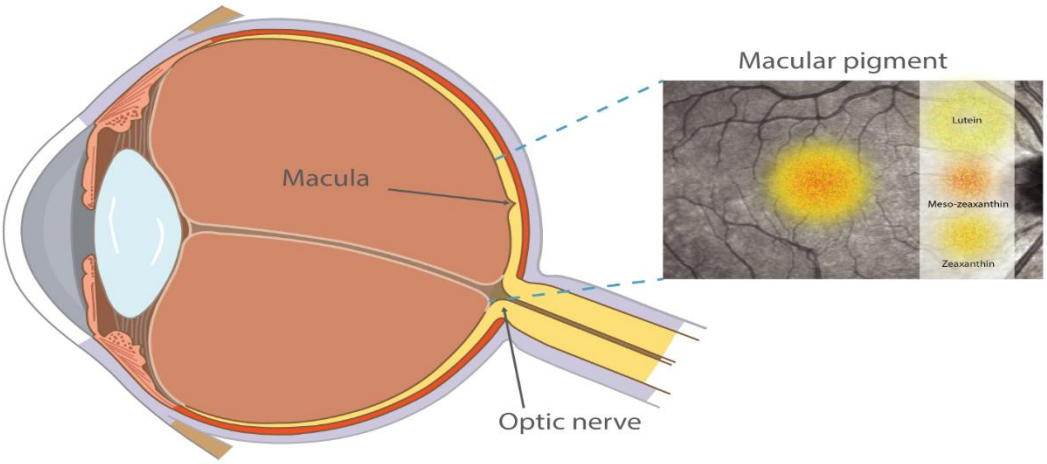


Figure 6.1: Diagram highlighting the location of MP and the macula within the eye.

Macular pigment has a number of important functions in the eye. Evidence suggests that MP contributes to visual performance and/or experience, as it acts as an optical filter for blue light (peak absorption ≈ 460 nanometres).[226, 2] The importance of MP's antioxidant and anti-inflammatory properties is supported by its capacity to protect against the cumulative damaging effects of oxidative stress,[291] and inflammation,[283] which affect the macula in conditions such as AMD.[5, 218, 259, 377] In light of this evidence, the relatively recent emphasis on MP as a possible ocular-protectant in diabetes,[6] a pathological condition similarly associated with inflammation, oxidative stress and progressive retinal damage; is logical and represents a natural extension of previous work in AMD.[5, 218, 259, 377]

The underlying molecular mechanisms associated with the onset of diabetes and the potential protective effects of lutein and/or zeaxanthin against retinal oxidative damage, inflammation and visual function have been explored in diabetic murine models.[14, 20, 186, 215, 378-380] The onset of diabetes in alloxan and STZ diabetic mice and rats was accompanied by an increase in markers of oxidative stress and inflammation including: malondialdehyde (MDA), 8-hydroxyguanosine (8-OHdG), NF-κB and VEGF, with a concomitant decrease in local antioxidants including GSH, and GPx.[14, 186, 215] Importantly, treatment with antioxidants (lutein and/or zeaxanthin) have been shown to lower oxidative stress and inflammation and increase endogenous antioxidants; findings which in some[186, 215] but not all [20] studies occurred independently of any effects on hyperglycaemia. The beneficial effects of these carotenoids on retinal function have also been observed in diabetic murine models**,** and these include the preservation of ERG b-wave amplitude and prevention of neurodegenerative effects on the inner retinal layers.[14, 186, 215] Findings from these

studies provide important insights into the potential role that MP may have in protecting against diabetic retinal disease, however, the observed findings need to be interpreted with caution, as higher MP dosages have been used in animal studies compared with those used clinically in humans, and many of these studies have never conclusively demonstrated retinal uptake of the administered carotenoids beyond the RPE and choroid.[381, 382] A summary of the experimental animal studies examining MPOD in diabetes is outlined in Table 6.1.

A number of cross-sectional studies,[6, 7, 9, 383, 384] including findings from our own study group,[7] and RCTs,[15, 21] have explored the relationship between Type 1 and Type 2 diabetes and MPOD in humans. The MPOD is generally found to be lower in diabetes [6, 7, 9] with some studies suggesting that oxidative stress is implicated in its depletion.[6, 9] Lower levels of MPOD have also been associated with raised HbA1c levels and the presence of retinopathy in patients with Type 2 diabetes.[6] In another study, patients with diabetes (Type 1 & 2) with grade 2 diabetic maculopathy, had significantly lower MPOD compared to those with no maculopathy (p=0.016).[9]

Although oxidative stress is implicated as a causative factor, the exact reasons why MP is lower in diabetes, and Type 2 diabetes in particular [6, 7] are not fully understood. One study found lower levels of MP in Type 2 versus Type 1 diabetes,[7] suggesting that other coincident metabolic and pathological abnormalities which are characteristic of Type 2 diabetes (e.g. overweight/obesity, dyslipidaemia), may explain the lower MPOD levels observed. Furthermore, lutein and zeaxanthin supplementation has been shown to exert positive and beneficial ocular effects in those with diabetic eye disease, including structural improvements in measures of macular

oedema and functional improvements in VA and other visual function measures.[15, 21] Table 6.2 outlines a summary of the cross-sectional studies and RCTs examining the relationship between MPOD and diabetes (Type 1 & 2).

Although the evidence exploring MP and diabetes is relatively sparse, the findings which have been reported are sufficient to suggest that MPOD may be adversely affected by the condition, and that there is a plausible rationale to explore the possible benefits of lutein and/or zeaxanthin supplementation for ocular health and visual function in diabetes, particularly Type 2. The recent generation of such evidence has prompted this literature review, which is designed to explore and elucidate the metabolic perturbations and candidate causal mechanisms which may underpin the likely complex interdependency of MP and diabetic eye disease.

Table 6.1: Summary of experimental animal studies examining the relationship between lutein and/or zeaxanthin and diabetes.

Author & Year	Design: Animal Model	Lutein and/or Zeaxanthin	Outcome, Evidence & Conclusion
Muriach et al (2006).[215]	Albino mice (alloxan induced diabetes mellitus).	Lutein (70% purity, 0.2 mg/kg body weight).	Decrease in oxidative stress (decrease MDA and NF-κB, increase GSH and GPx); ERG b-wave restored.
Kowluru et al (2008).[14]	Rats (STZ induced diabetes mellitus).	Zeaxanthin (0.02% or 0.1% equivalent to 8.4 mg/kg). Placebo controlled.	Decrease in oxidative stress (decrease 8-OHdG, nitrotyrosine, iNOS, VEGF & ICAM-1 levels).
Arnal et al (2009).[380]	Rats (STZ induced diabetes mellitus).	Lutein (in combination with DHA). Placebo controlled.	Decrease in oxidative stress (MDA and nitrotyrosine, increase GSH & GPx). Restored ERG b-wave amplitude & latency time.
Sasaki et al (2010).[186]	Mice (STZ induced diabetes mellitus).	Lutein (0.1 mg wt/wt). Placebo controlled.	Decrease in ROS & ERK activation and apoptosis. Protection of inner retina from visual impairment.
Tang et al (2011).[378]	Mice (db/db) spontaneous diabetes.	1% (kcal) Wolfberry (lutein & zeaxanthin). Placebo controlled.	Lower expression of endoplasmic reticulum stress biomarkers, (BiP, PERK, ATF6, caspase-12) and restored AMPK, thioredoxin, Mn-SOD, and FOXO3α activities.
Yu et al (2013).[379]	Mice (db/db) spontaneous diabetes.	Wolfberry diet (lutein and zeaxanthin). Placebo controlled.	Activation of AMPK in mitochondria which caused retinoprotection in the retina of db/db mice.
Kowluru et al (2014).[20]	Rats (STZ induced diabetes mellitus).	Multi-nutritional supplement, lutein=20 mg; zeaxanthin=40 mg/kg of powder diet, in addition	Carotenoids ameliorated capillary cell apoptosis. Improved ERG, a- and b- wave amplitudes. Decrease oxidative damage & increased antioxidant activity. Inflammatory markers (decrease

		to numerous antioxidants. Placebo controlled.	in VEGF, IL-1β & NF-kB).
Zhou et al (2017).[385]	Rats (High fat diet and low dose STZ induced diabetes mellitus).	Zeaxanthin (50 mg/kg; 200 mg dissolved in 10 ml corn oil). Placebo controlled.	Supplementation with zeaxanthin reduced blood glucose, improved cognitive deficits, neural cell survival and increased p-AKT levels. Inhibited cleaved caspase-3 levels and NF-κB nuclear transcription in the hippocampus.

Abbreviations: mg, milligram; MDA, malondialdehyde; NF-κB, nuclear factor κ B; GSH, glutathione; GPx, glutathione peroxidase; ERG, electroretinogram; STZ- streptozotocin; 8-OHdG, 8-hydroxy-2[1] – deoxyguanosin; iNOS, inducible nitric oxide synthase; VEGF, vascular endothelial growth factor; ICAM -1, intercellular adhesion molecule; DHA, docosahexaenoic acid; ROS, reactive oxygen species; ERK, extra cellular receptor kinase; kcal, kilocalorie; BiP, binding immunoglobulin protein; PERK, protein kinase RNA-like ER kinase; ATF6, activating transcription factor 6; AMPK, AMP-activated protein kinase; Mn SOD manganese superoxide dismutase; FOXO3α, forkhead O transcription factor 3 α; IL-1 β, interleukin 1 beta; p-AKT; phosphorylated serine/threonine kinase.

Table 6.2: Summary of cross-sectional studies and RCTs examining the relationship between MPOD and diabetes.

Author & Year	Design	Outcome, Evidence & Conclusion
Davies & Morland (2002).[9]	Cross-sectional study to assess MPOD in diabetes (N = 26) vs healthy, non-diabetic controls (N = 30).	Those with maculopathy had lower levels of MP than those without maculopathy (*p* = 0.016).
Zagers et al (2005).[383]	Cross-sectional study to assess MPOD in diabetes (N=15) vs healthy non-diabetic subjects (N=14).	The density of MP in diabetic participants was not different from that in controls (*p* = 0.3).
Mares et al (2006).[384]	Cross-sectional study on sub-sample of women who participated in CAREDS (n = 1698). MPOD measured by HFP.	Lower MPOD was associated with diabetes. Relationship of dietary intake of lutein and zeaxanthin to MP differed between diabetic and non-diabetic women.
Lima et al, (2010).[6]	Cross-sectional study including: non-diabetic controls (N=14), diabetic patients with (N=12) and without retinopathy (N=17).	Type 2 diabetic patients, with or without retinopathy, had reduced MPOD vs. non-diabetic subjects. MPOD was inversely correlated with HbA1c levels.
Scanlon et al, (2015).[7]	Cross-sectional study assessing MP in Type 1 (N=34) & 2 (N=68) diabetic participants and healthy controls (N=48).	MPOD levels were lower in diabetic participants vs controls (*p* =0.04). MPOD was lower in Type 2 vs Type 1 (*p* = 0.02) and Type 2 vs controls (*p* = 0.03).
She et al, (2016).[386]	Cross-sectional study assessing MP in diabetic patients without retinopathy (N=134), with early stage non-proliferative diabetic retinopathy (N=48) and non-diabetic controls (N=219).	MPOD levels were not significantly different between the three groups (*i.e.* diabetic participants with or without retinopathy and controls (p = 0.24). MPOD was positively associated with central foveal thickness (*p*=0.001).

Moschos et al, (2017).[387]	Retrospective study on Type 2 diabetic patients (N=60; 120 eyes) without diabetic retinopathy. Patients received carotenoid supplements containing lutein (10 mg), zeaxanthin (2 mg) and *meso*-zeaxanthin (10 mg) once a day for two years.	MPOD was not measured in this study. OCT showed an increase in the central foveal thickness (p<0.001) and mf-ERG revealed increased retinal response density within the central 13° surrounding the fovea (rings 1 to 3) (p<0.001) at 2 years after the onset of carotenoids supplement intake.
Gonzalez-Herrero et al, (2018).[388]	Prospective controlled study assessing macular integrity and macular sensitivity by microperimetry in patients with non-proliferative diabetic retinopathy. Patients were assigned (1:1) to the DHA + xanthophyll supplementation group (N=12; 24 eyes) or the control group (N=12; 24 eyes).	Significant improvements in macular sensitivity at 90 days (p=0.030) in the supplemented group. Total antioxidant capacity levels increased and plasma IL-6 levels decreased significantly in the DHA + xanthophyll supplemented group but not in the control group. MPOD was not measured in this study.
Cennamo et al, (2019).[389]	Prospective controlled study assessing MPOD and microvascular density on OCTA in a cohort of Type 1 diabetes patients with retinopathy (N=59, total of 82 eyes). The control group (N= 40; total of 80 eyes examined).	MPOD was significantly lower in patients with retinopathy compared with controls (p<0.001). OCTA vessel density were significantly lower in diabetes patients than in controls (p<0.001).
Hu et al, (2011).[15]	RCT: Controlled trial including non-proliferative diabetic retinopathy patients (N=61) & non-diabetic controls (N=60).	Plasma lutein/zeaxanthin concentrations in diabetic retinopathy patients were lower than those in normal controls. Lutein/zeaxanthin intake can improve VA, contrast sensitivity & macular oedema in diabetic retinopathy patients.

Chous et al, (2015).[21] DiVFuSS	RCT: Type 1 (N=27) and Type 2 (N=40) diabetes with no diabetic retinopathy or mild to moderate non-proliferative diabetic retinopathy versus placebo. Intervention group received 2 daily doses of a xanthophyll, antioxidant and plant extract supplement.	Significant improvement in MPOD (*p* values: 0.008 to <0.0001); visual function on all measures (*p* values: 0.008 to <0.0001); plasma lipids (*p* values ranging from 0.01 to 0.0004), hs-CRP (*p*=0.01) and diabetic peripheral neuropathy (Fisher's exact test, *p*=0.0024). These effects were observed without an effect on glycaemic control.

Abbreviations: MPOD, macular pigment optical density; MP, macular pigment; CAREDS, carotenoids in age related eye disease study; HFP heterochromatic flicker photometry; HbA1c, glycated haemoglobin; DR, diabetic retinopathy; OCT, optical coherence tomography; mfERG; multifocal electroretinography; DHA, docosahexaenoic acid; IL-6, interleukin 6; OCTA, optical coherence tomography angiography; RCT, randomised control trial; VA, visual acuity; DiVFuSS, the diabetes visual function supplement study; hs-CRP, high sensitivity – C reactive protein.

6.3 Literature search methods

For the purpose of this review, partly because of the relative dearth of higher level RCT evidence, we have appraised all forms of published research, even lower level evidence sources, to ensure that the scientific and clinical implications of the available evidence might be synthesised into useful treatise, which accurately reflects what is known about this area, and what remains to be elucidated. All relevant published articles from human and animal studies which reported on the relationship between MP and diabetes (Type 1 & Type 2), were identified for the first stage of the review, up until the year 2019. Secondly, the review focused on identifying publications which investigated the relationship between the metabolic perturbations typically associated with diabetes, and Type 2 diabetes in particular (e.g. adiposity/dyslipidaemia) and MP. Pre-selected keywords including: 'lutein', 'zeaxanthin', 'macular pigment', 'diabetes', 'diabetes AND MP', 'diabetes AND lutein/zeaxanthin', 'BMI', 'body fat AND diabetes', 'adipose', 'high density lipoprotein AND diabetes, 'triglycerides AND diabetes', 'oxidative stress', 'inflammation', 'hypertension AND MP', 'insulin resistance', 'hyperinsulinemia', were entered into academic databases and search engines including PubMed, Google Scholar, Mendeley, Scopus, Cochrane Library and the ISRCTN registry to define our search of the literature relating to MP and diabetes up until 2019. Although many supporting publications were retrieved, in total, only eight animal studies (Table 6.1) and eleven human studies (Table 6.2) were included to help clarify our current understanding of the links between diabetes and MP. A total of thirteen papers on adiposity and MP (Table 6.3) and seven papers on MP and dyslipidaemia (Table 6.4) were included to help analyse the relationship between MPOD and the metabolic correlates of Type 2 diabetes (adiposity and dyslipidaemia).

The overall findings suggest that MP is lower in diabetes, Type 2 diabetes in particular; and that supplementation with macular carotenoids and/or other antioxidants may confer protection against diabetic eye disease. To elucidate the causal mechanisms and metabolic perturbations which might possibly explain the lower MP levels observed in diabetes, we will first explore the condition diabetes mellitus itself, including its association with oxidative stress and inflammation; and subsequently present the evidence linking adiposity and dyslipidaemia with Type 2 (or poorly controlled Type 1) diabetes and MPOD.

6.4 Diabetes mellitus

Diabetes mellitus is a group of metabolic disorders caused by the complex interaction of genetics, environmental factors and lifestyle choices.[390] Diabetes is characterised by a deficiency of insulin and/or systemic insulin resistance. Over the past number of decades, the number of people with diabetes, particularly Type 2 diabetes, has increased dramatically, making it a critical and universal public health challenge.[391] Type 1 diabetes usually develops in normal-weight children, teenagers and younger adults, and is an autoimmune condition involving the selective destruction of pancreatic β cells, ultimately resulting in complete deficiency of insulin.[392] Conversely, Type 2 diabetes is linked to a sedentary lifestyle and being overweight, and is characterised by systemic insulin resistance and subsequent pancreatic endocrine dysfunction, which results in attenuated insulin synthesis as well as inhibition of its cellular effects.[390] Whilst diabetes is characterised by having higher than normal blood glucose levels, the pathogenesis and development of Type 1 and Type 2 diabetes differ; therefore, the relationship between MPOD and the different forms of diabetes should not be generalised. At presentation, Type 2 diabetes is most

often accompanied by other co-morbidities including overweight/obesity, insulin resistance, hypertension and dyslipidaemia, features which are less common in Type 1 diabetes at diagnosis, but which may occur as complications of Type 1 diabetes later in the course of the disease. Oxidative stress and inflammation are implicated in both Type 1 and Type 2 diabetes; however, research has shown that these metabolic disturbances are very pronounced in Type 2 diabetes.[393-397] The oxidative stress, inflammation, adiposity and dyslipidaemia which characterise diabetes, may have independent relationships with MPOD, and therefore constitute plausible causal mechanisms in diabetic eye disease. These pathological mechanisms merit further exploration and will be discussed in more detail herein.

6.4.1 Oxidative stress and diabetes

Chronic hyperglycaemia induces oxidative stress in patients with diabetes.[398] Oxidative stress, defined as the excessive production of ROS, results in oxidative injury when the redox balance is upset; *i.e.* where the level of oxidative species exceeds the capacity of the anti-oxidant defence system to neutralise them.[355, 399] Reactive oxygen species can include free radicals, which are partially reduced oxygen species containing one or more unpaired electrons (for example, superoxide anion or hydroxyl radical); and species with their full complement of electrons in an unstable or reactive state (for example, singlet oxygen or hydrogen peroxide).[400] The body's natural defence against oxidative damage is neutralisation by endogenous antioxidants,[401] which include enzymatic antioxidants such as SOD, CAT and GPx, and non-enzymatic antioxidants such as GSH. Our endogenous antioxidant defence system, however, is incomplete without exogenous antioxidant nutrients including vitamin C, vitamin E, carotenoids (beta carotene, lutein, zeaxanthin & *meso*-

zeaxanthin) and polyphenols.[177] Exogenous antioxidants, such as those available in dietary fruit and vegetables, are therefore necessary to balance redox status. Through normal physiological processes, antioxidants inhibit or quench free radical reactions and can delay or inhibit cellular damage.[399] When these processes are overwhelmed, however, as in Type 2 diabetes, ROS will readily react with lipids, proteins and nucleic acids, resulting in impaired cell function or cell death.[402-404]

The retina is already at an increased risk of oxidative stress and damage, given its high oxygen demand and consumption, its exposure to visible light irradiation and its high concentration of PUFAs in the photoreceptor outer segments.[405] These risks increase when the generation of free radicals and ROS is stimulated by environmental factors,[406, 407] short-wavelength light exposure,[408] and other internal stresses including inflammation,[409] and hyperglycaemia.[403, 404, 410] While PUFAs in the photoreceptor outer segment are thought to protect against oxidative and inflammatory damage, they can potentially act as a substrate for the propagation of free radicals as they provide a readily available source of electrons.[291, 406] With increasing age, systemic antioxidant levels decline and ROS levels increase in most tissues, and this is associated with a number of neurodegenerative diseases, including AMD and diabetes.[291, 411,9]

Hyperglycaemia, oxidative stress and changes in redox homeostasis are fundamental events in the pathogenesis of diabetic retinopathy. Various mechanisms have been suggested to contribute to the formation of reactive oxygen-free radicals. Hyperglycaemia causes tissue damage through five major pathways, namely increased flux of glucose and other sugars through the polyol pathway; increased intracellular formation of AGEs; increased expression of the receptor for AGEs (RAGEs) and its

activating ligands; activation of PKC isoforms; and activity of the hexosamine pathway.[412] Over activation of these pathways can lead to damage of cellular organelles and enzymes, increased lipid peroxidation, and development of insulin resistance.[413, 414] Several lines of evidence indicate that all five mechanisms are activated by a single upstream event: mitochondrial overproduction of ROS.[415] Locally, the occurrence of these metabolic changes, results in defective angiogenesis and the activation of a number of pro-inflammatory pathways which stimulate an influx of leukocytes which can alter vascular permeability in the retina.[378] The role of hyperglycaemia in diabetic microvascular complications in the eye has been established by large-scale prospective studies in Type 2 diabetes (UKPDS).[416] Beyond elevations in blood glucose, however, ROS formation is also increased by elevations in FFAs (a common feature of Type 2 diabetes), through their direct effect on mitochondria.[417]

While, oxidative stress is increased in diabetes, the activities of antioxidant defence enzymes responsible for scavenging free radicals and maintaining redox homeostasis (SOD, CAT, GPx) are concomitantly diminished in the retina of both Type 1 and Type 2 diabetes.[355, 401, 418] The activity of non-enzymatic antioxidants is also depressed during hyperglycaemia-induced oxidative stress.[418] Redox balance is important, as its disruption can cause antioxidants to become pro-oxidative, potentially precipitating damage to the retina. It cannot be assumed, however, that a single reductive or antioxidant treatment would be sufficient to reverse the underlying pathophysiological mechanisms of diabetic retinopathy. Antioxidant protection in the retina is provided mainly from vitamins C and E, carotenoids, GPx, SOD and CAT enzymes, and GSH compound,[419] and many of these antioxidants are aided by micronutrients (e.g. copper,

zinc, selenium) which act as cofactors.[420] The carotenoids lutein and zeaxanthin are very effective quenchers of singlet molecular oxygen and lipid peroxy radicals, however, in the process, these carotenoids are themselves oxidised to their corresponding radical cations.[250] These cations must be reduced to regenerate the original carotenoid, thereby allowing their reuse as an antioxidant. Vitamin E (α-tocopherol) reduces oxidised carotenoids, which leaves tocopherol oxidised.[420] However, oxidised tocopherol can be reduced and regenerated by vitamin C (ascorbic acid) and GSH, which are themselves further reduced by copper and zinc.[5, 421] Dietary lutein, zeaxanthin and vitamins E and C, as well as endogenous antioxidant enzymes, therefore, act in concert with one another to lower the levels of ROS (via ROS detoxification) and to recycle oxidised lipid antioxidants (via re-reduction).

The presence of oxidation products of the macular carotenoids in human and primate retinal tissue indicates that lutein, zeaxanthin and *meso*-zeaxanthin play an important antioxidant role there.[45] Zeaxanthin appears to be a more potent anti-oxidant than lutein,[270] with *meso*-zeaxanthin being even more efficacious, but only in conjunction with its binding protein.[271] Li *et al.* [272], demonstrated that a 1:1:1 ratio mixture of lutein, zeaxanthin and *meso*-zeaxanthin quenches more singlet oxygen than any of these carotenoids individually at the same total concentration,[272, 422] suggesting that they are most effective when acting synergistically with one another. Diet is a major lifestyle factor which can greatly influence the incidence and progression of Type 2 diabetes. Some dietary and lifestyle modifications associated with enhanced antioxidant supply could therefore be an effective prophylactic means to prevent or limit oxidative stress in diabetic eye disease. Type 2 diabetes is associated with a sedentary lifestyle and being overweight, therefore, patients with Type 2 diabetes may

not only be exposed to diets which are high in fat and low in antioxidants,[423] and to the increased oxidative stress imposed by their diabetes;[6, 7, 9] but may also be subject to greater chronic oxidative stress as a result of their obesity itself.[424] The co-occurrence of these phenomena consequently demands an even greater supply of exogenous antioxidants (vitamin C, E, carotenoids lutein, zeaxanthin & *meso*-zeaxanthin) to balance redox status and to combat this state of chronic oxidative stress to which the diabetic retina is exposed. While the control of blood glucose is critical in patients with diabetes, neuroprotective strategies and novel antioxidant treatments should also be considered to target the specific aetiologies and underlying pathophysiological mechanisms of the condition, and to ultimately protect against the vascular pathologies of diabetes. It is logical that natural antioxidants (lutein, zeaxanthin and *meso*-zeaxanthin) may have beneficial implications for diabetic retinopathy in this context. However, larger trials involving intensive assessment of oxidative stress, antioxidant defence activities and their effects on one another in the diabetic state are necessary before empirical supplementation of these carotenoids can be recommended clinically.

6.4.2 Inflammation and diabetes

Diabetic retinopathy has traditionally been considered a disease of the retinal microvasculature, associated with oxidative stress induced by hyperglycaemia. More recently, however, inflammation has been cited as a deleterious factor in the development of diabetic complications,[10] and is now also implicated in the pathogenesis of many ocular diseases including AMD, [425] and diabetic retinopathy.[426] Numerous interplays exist between inflammation and oxidative stress and vice versa.[427] Hyperglycaemia increases the levels of pro-inflammatory proteins,[10] and it is

now believed that inflammatory processes underlie many of the functional changes in retinal vasculature observed histologically in early diabetic retinopathy, such as pericyte loss, microaneurysms, and occluded and degenerated capillaries.[194]

While inflammation is normally a protective response to tissue stress, hyperglycaemia leads to a dysregulation of inflammation, which ultimately becomes chronic. Oxidative stress triggers inflammatory responses, and inflammation also enhances the production of ROS, which creates a self-perpetuating cascade towards oxidative tissue damage.[428] The increased expression of many inflammatory proteins in diabetic retinopathy is regulated through the activation of pro-inflammatory transcription factors including NF-κB.[429] Activation of NF-κB leads to the synthesis of pro-inflammatory molecules such as IL-1, IL-6, TNF-alpha, c-Jun N-terminal kinase-1 (JNK-1), inhibitor of nuclear factor kappa-B kinase subunit beta (IKK-β) and growth factors such as VEGF.[378, 429]

In the retina, the microvascular endothelium forms an effective barrier to control the movement of blood, fluid and proteins across the vessel wall. This barrier is formed by tight junctions, which consist of over 40 different proteins and various inflammatory mediators including VEGF, TNFα, PKC, IL-1β, and IL-6.[194, 430] Dysfunction of these proteins is highly correlated with the pathogenesis of blood-retinal barrier breakdown and increased vascular permeability, which leads to diabetic macular oedema, a major cause of vision loss in diabetic retinopathy.[431] Inflammatory processes can induce oxidative stress, and similarly, oxidative stress can induce inflammation through the activation of multiple pathways. The coincident over-activation of inflammation and oxidative stress can therefore deplete cellular

antioxidant capacity and reduce MP levels in the eye.[4, 291]

The expression of various pro-inflammatory cytokines, such as TNF-α, IL-1 and IL-6, are also increased from visceral adipose tissue (more common in Type 2 diabetes), and this has been linked to systemic inflammation and accompanying insulin resistance.[432] The inhibition of signalling downstream of the insulin receptor is a primary mechanism through which inflammatory signalling leads to insulin resistance.[10] Several cross-sectional studies have shown that insulin resistance and Type 2 diabetes are associated with higher levels of C-reactive protein (CRP), IL-6 and TNF-alpha, all markers of subclinical inflammation.[433-435] Various longitudinal studies have also shown that elevated levels of CRP and IL-6 are linked to the later development of Type 2 diabetes.[436, 437]

Inhibition of different inflammatory mediators, including NF-kβ, IL-1β, VEGF and cyclooxygenase-2 (Cox-2), has been shown to limit the degeneration of retinal capillaries characteristic of early stage diabetic retinopathy.[438] There is evidence to suggest that lutein and zeaxanthin may prevent the development of diabetic retinopathy by suppressing ROS induced by inflammation.[377] Li *et al.* [283] investigated the anti-inflammatory effects of lutein on Muller cells in a murine model. Treatment with lutein led to lower production of the pro-inflammatory factors NF-kB, IL-1β and Cox-2, suggesting an anti-inflammatory role for lutein in protection against retinal ischemic/hypoxic injury.[283] In another study, the protective effects of lutein and zeaxanthin against photo-oxidative damage to RPE cells were investigated. Supplementation with carotenoids modulated the inflammatory responses in these cultured RPE cells in response to photo-oxidation.[439]

The effects of zeaxanthin on diabetes induced retinal oxidative damage and inflammation have also been investigated in diabetic rats.[14] It was found that zeaxanthin significantly inhibits diabetes-induced retinal oxidative damage and elevations in VEGF and its adhesion molecule, abnormalities which are commonly associated with the pathogenesis of diabetic retinopathy.[14] Clinical findings have highlighted the inverse association between lutein and IL-6 in coronary artery disease patients.[440] A recent novel RCT, the diabetes visual function supplement study (DiVFuSS),[21] explored the effects of a nutritional supplement (which included lutein and zeaxanthin) on patients with Type 1 and Type 2 diabetes, and suggested that the preparation used mitigated the damaging effects of systemic inflammation on ocular function, and that these effects may have been mediated by enhancements in MPOD. Chronic low grade inflammation and oxidative stress co-exist in diabetes, particularly Type 2 diabetes but also poorly controlled Type 1 diabetes. Therefore, findings from DiVFuSS, which achieved significant increases in MPOD (27% mean increase in DiVFuSS group vs. 2% mean decrease in placebo group), as well as reductions in plasma high-sensitivity (hs)-CRP (60% mean decrease in DiVFuSS group vs. 11% mean decrease in placebo group), may be due in part to the inclusion of compounds which in combination, specifically target both inflammation and oxidative stress, and therefore, which block some of the oxidative and/or inflammatory pathways responsible for the progression of diabetic retinopathy.[21] The inclusion of many compounds in this test formula and the fact that both types of diabetes patients (Type 1 & 2) were examined renders interpretation of the outcome more difficult, however, these findings are promising as they clearly show that MPOD can be augmented in patients with Type 2 diabetes.[21]

While it is clear that oxidative stress and inflammation are implicated in the initiation and development of diabetic retinopathy, it remains unclear whether the pathological effects of oxidative stress/inflammation are mediated through MPOD depletion in Type 2 diabetes. The current body of evidence which directly links lower MP levels with other metabolic correlates of diabetes, namely adiposity and dyslipidaemia; will now be explored to elucidate the possible mechanisms by which these metabolic factors mediate their effects on MPOD. It is important to note that both increased adiposity and dyslipidaemia (primarily reduced HDL) may adversely affect MP by compromising the availability,[16, 17, 54, 441] and transport,[18, 19] of dietary carotenoids to the retina. It follows that a condition such as Type 2 diabetes, which unfavourably affects these parameters, may have deleterious effects on MPOD, with clinically relevant outcome effects. An analysis of the literature is presented describing these putative mechanisms: (a) evidence in relation to MPOD and body fat/adiposity (n =13, Table 6.3); and (b) evidence concerning MPOD and dyslipidaemia (n =7, Table 6.4).

6.4.3 Type 2 diabetes, MPOD & adiposity – the evidence.

Diabetes mellitus is a chronic disorder which can alter carbohydrate, protein, and fat metabolism. Weight gain, following excessive calorie intake, results in the body becoming markedly resistant to the action of insulin. Despite initial impairment in insulin action, glucose tolerance can remain normal for some time because there is a compensatory increase in insulin secretion by pancreatic β-cells, which results in a well-compensated metabolic state, a condition known as hyperinsulinemia. There is now mounting evidence to indicate that persistently elevated plasma insulin levels can contribute to the development of hypertension, plasma lipid abnormalities and atherosclerosis.[442] The escalating rates of insulin secretion as a result of advancing

obesity, however, cannot be maintained and Type 2 diabetes ensues.

Intra-abdominal fat appears to be an important determinant of insulin sensitivity compared with subcutaneous fat.[443] Visceral and subcutaneous fat differ in their phenotypic, physiological and functional aspects, as there are a greater number of macrophages, T lymphocytes and pro-inflammatory molecules in visceral versus subcutaneous fat in obese individuals. Abdominal adipose tissue produces a variety of adipocytokines including IL-1, IL-6, IL-8, TNF-alpha, leptin and resistin, therefore, the abdominal adipose mass acts as an important mediator of inflammation in diabetes.[444] Obesity also leads to an increased size of adipocytes. The death of adipocytes, which is rare in healthy people, is very common in obese individuals and has been linked to adipocyte hypoxia. The accumulation of macrophages in the adipose tissue of obese individuals, not only eliminates the dead cells but also further increases their synthesis of inflammatory mediators IL-8, IL-6, IL-1, TNF-alpha.[444] The resultant inflammation becomes chronic, therefore, obesity is considered a low-grade inflammatory condition which in turn leads to an increase in oxidative stress. Plasma 8-hydroxy-2[1]–deoxyguanosin (8-OHdG), a known sensitive marker of oxidative DNA damage and of total systemic oxidative stress *in vivo*, has been shown to be positively correlated with BMI in people with Type 2 diabetes mellitus.[370] Over-expression of oxidative stress and the concomitant reduction in antioxidant defence, damages cellular structures,[367] often resulting in further inflammatory responses. Antioxidant defences may be lower in overweight/Type 2 diabetes patients, due to their lower intake of antioxidant rich foods (e.g. fruits and vegetables), their increased utilisation of these molecules (e.g. increased inflammation/ROS in insulin dependent tissue such as the retina) and their impaired generation of other supportive anti-

oxidants,[367] factors which may collectively lead to MPOD depletion.

The diminished response to insulin in Type 2 diabetes also leads to increased lipolysis in adipocytes. Enlarged adipocytes release FFAs and adipocytokines,[418, 442] therefore, greater amounts of FFAs reach the liver via the portal vein, resulting in fatty liver infiltration. Mobilised FFAs are oxidised by vascular endothelium to generate ROS. Elevations of FFAs in plasma also inhibit insulin suppression of hepatic glucose production, leading to an increase in glucose production by the liver.[445] This in turn, results in both insulin resistance and inflammation in the major insulin target tissues (skeletal muscle, liver and endothelial cells). Abnormal levels of lipids, fatty acids, and various adipocytokines from adipose tissue therefore initiate a vicious cycle of fat damage, inflammation, worsening insulin resistance, impaired β-cell insulin secretion and ultimately Type 2 diabetes.[442, 446] The chronic low grade inflammation, associated with obesity and Type 2 diabetes leads to increased oxidative stress and further inflammation, putting a greater demand on antioxidant defences.

Apart from its pro-inflammatory and pro-oxidant effects, adipose tissue is also a major body store for carotenoids such as lutein and zeaxanthin. Higher levels of body fat have been shown to be related to lower levels of circulating carotenoids,[347] making these pigments less available to retinal tissue.[68] Furthermore, a randomised controlled weight loss trial found that a significant weight loss in the intervention group was related to increases in plasma lutein.[447] Higher BMI [448] and higher body fat percentage [16] have both been linked with an increased risk of AMD. Studies have reported an inverse relationship between adiposity and BMI and MPOD,[52, 54] with the observed relationships largely attributable to participants with a BMI > 29 kg/m^2.[54] One study

found that higher body fat percentage, even within relatively healthy limits, was associated with lower retinal tissue lutein and zeaxanthin status,[53] suggesting that adiposity may affect the nutrient status of the retina. Gender differences in MPOD have also been reported,[16, 17] which may imply differences in metabolism of MP, or reflect differences in adipose tissue storage of these dietary carotenoids between men and women. Competitive absorption by a larger overall fat mass (percentage fat mass is generally higher in women),[449] or specifically by greater abdominal fat mass (which is more common in men),[450] may reduce lutein and zeaxanthin uptake by the retina. Concentrations of carotenoids differ according to body site, with levels demonstrably higher in abdominal fat,[67] which may explain the differences in MPOD currently observed between men and women.[16, 17] Since adipose tissue may trap lutein and zeaxanthin [53] individuals who are overweight or obese (Type 2 diabetes) may consequently have lower concentrations of carotenoids lutein and zeaxanthin available at the macula.

Overall the research appears to suggest that obesity has a negative impact on MPOD, however, there is also some evidence to the contrary. One study found that mean MPOD values did not differ significantly between subjects with various types of obesity and ideal weight subjects.[364] Our previous study [7] is the only one to date which has examined the relationship between BMI and MPOD in a group with diabetes. This study found that MPOD was significantly lower and BMI was significantly higher in patients with Type 2 diabetes, compared to Type 1 and non-diabetic controls, highlighting the possibility that different relationships exist between MPOD status and Type 1 and 2 diabetes. The higher BMI levels observed in Type 2 diabetes are reflective of excess adiposity which acts as a store for lutein and zeaxanthin. However,

as described previously, obesity is also independently associated with increased oxidative stress,[69, 367, 368] and with inflammation [372] which exacerbates oxidative stress, and may therefore have multiple depletive effects on MP. Unfortunately the cross-sectional nature of this study [7] means that it cannot elucidate the extent or nature of any causal relationships between BMI and MPOD in these Type 2 subjects.

The different presenting features of Type 1 and 2 diabetes and the interplay of their disease components with MPOD status (e.g. adiposity), may mean that the mechanistic relationship between these conditions and MPOD status, is not uniform across both. The fact that much of the research to date includes both diabetic groups may obfuscate any relationships present. Although, the evidence to date is somewhat conflicting, there appears to be a theoretical basis for a relationship between the characteristic body fatness of Type 2 diabetes and lower levels of MPOD. A summary of the studies investigating the association between MPOD and adiposity are outlined in Table 6.3.

Table 6.3: Summary of studies linking MPOD and body fat.

Author & Year	Design	Sample	Results
Johnson et al (2000).[17]	Study 1: 15 week modified diet to increase lutein & zeaxanthin intake with plasma, buccal mucosal cell and adipose samples taken at 0, 4, 8 & 15 weeks and 2 months post-intervention. Study 2: A cross-sectional analysis of relationships.	1. N = 7 (4 women & 3 men). 2. N = 21 (13 women & 8 men).	1. Only plasma zeaxanthin increased from baseline and only at week 4. Adipose tissue lutein peaked at week 8. 2. Significant negative correlations found between adipose tissue, lutein concentrations and MP for women. A positive effect was seen in men.
Broekmans et al (2002).[63]	Cross-sectional design looking at plasma and adipose tissue concentrations in relation to MP.	376 healthy adults. Plasma (N=376) Adipose (N=187)	Regression models showed a positive significant association between MP density and plasma lutein, plasma zeaxanthin, and adipose lutein concentrations in men after adjustment for age, but no relation in women. This effect remained in men after further correction.
Hammond et al (2002).[54]	Cross-sectional study investigating MPOD, BMI, body fat percentage, dietary intake and plasma carotenoid concentrations.	680 healthy adults. Body fat (N=400) Dietary (N = 280) Plasma (N = 280)	Inverse association between MPOD and BMI ($p < 0.0008$) and body fat ($p < 0.01$).
Nolan et al	Cross-sectional study investigating MPOD,	100 healthy adults.	Significant inverse relationship between MPOD and body

(2004).[16]	body fat percentage, dietary carotenoid intake and plasma levels.	Males (N= 45) Females (N=55)	fat percentage in males (p<0.05) but not in females (p=0.14).
Mares et al (2006).[384]	Cross-sectional study investigating MPOD, dietary intake of lutein & zeaxanthin and other predictors of MP levels.	Subsample of women who participated in CAREDS. N=1698 women aged 53-86 years.	MPOD is directly related to dietary intake and plasma levels of lutein & zeaxanthin. Higher abdominal fat is related to lower MPOD.
Moeller et al (2009).[451]	Intervention in a sub-sample of the Women's Health Initiative to increase lutein and zeaxanthin through dietary modification.	394 healthy adults. Intervention (N = 1581) Control/usual diet (N = 236).	Lower MPOD was associated with above median WC. This explained 2.7% of the variation.
Kirby et al (2011). [447]	Randomised controlled weight loss trial.	104 subjects with BMI >28 kg/m^2; Intervention group (N= 54); Control group (N= 50).	Significant weight loss in the intervention group (p<0·0001), was related to plasma increases in lutein.
Gupta et al (2012).[364]	Clinical study; MPOD was compared to various anthropometric measurements including WHtR, WC, WHpR and BMI.	161 healthy adults. Males (N= 82) Females (N= 79).	Mean MPOD values did not differ significantly in various types of obesity when compared with normal subjects.
Nolan et al (2012).[52]	Prospective cohort study.	Subset of TILDA participants with MPOD &	Obese individuals had significantly lower MPOD (mean = 0.190) than either overweight individuals (mean =

		BMI measures N = 4281 adults aged over 50.	0.208; $p < 0.01$) or those with normal weight (mean = 0.213; $p < 0.01$).
Bovier et al (2013).[53]	Cross-sectional study investigating MPOD, assessment of body composition and plasma lutein/zeaxanthin status.	100 healthy adults. Males (N=39) Females (N=61).	Significant inverse relationship between body fat percentage (total and regional) and MPOD (p<0.01).
Scanlon et al (2015).[7]	Cross-sectional study to assess MPOD in Type 1 and Type 2 diabetic participants and BMI, dietary intake and plasma carotenoid concentrations.	102 diabetic participants, of which Type 1 (N=34) & Type 2 (N=68), and 48 healthy controls.	BMI was significantly higher and MPOD lower in Type 2 vs Type 1 diabetics and healthy controls. BMI was not directly correlated with MPOD.
Khan et al (2018).[452]	Cross-sectional study investigating MPOD, whole body adiposity (% Fat) and intellectual ability.	114 adults with overweight and obesity (≥25 kg/m^2).	MPOD was inversely related to body fat % (p=0.04) and positively associated with IQ (p=0.01) and fluid intelligence (p=0.01).
Edwards et al (2019).[453]	Cross-sectional study investigating MPOD, body fat % (DXA) and event related brain potentials.	101 adults with overweight and obesity (≥25 kg/m^2).	MPOD was not significantly associated with body fat % in this study (p>0.07). MPOD was associated with mean and peak amplitude (N2) (p<0.04) and latency (P3) (p<0.01) of neuroelectric indices.

Abbreviations: MPOD, macular pigment optical density; MP, macular pigment; BMI, body mass index; CAREDS, carotenoids in age-related eye disease study; kg/m^2 , kilogram per metre squared; WC, waist circumference; WHtR, waist-to-height ratio; WHpR, waist-to-hip ratio; TILDA, the Irish longitudinal study of aging; IQ, intelligence quotient; DXA, dual-energy X-ray absorptiometry.

6.4.4 Type 2 diabetes, MPOD and dyslipidaemia – the evidence

Insulin resistance and obesity, metabolic abnormalities commonly associated with Type 2 diabetes, may themselves lead to disturbances in the production and clearance of plasma lipoproteins.[158] Dyslipidaemia is often characterised by low levels of HDL and hypertriglyceridemia. In addition, LDL particles are converted to smaller, more atherogenic lipoproteins.[160] A number of factors are likely to be responsible for diabetic dyslipidaemia and these include insulin's effects on liver apoprotein production, dysregulation of LPL, impaired action of CETP and the peripheral action of insulin on adipose and muscle.[158] Hormone sensitive lipase and LPL are two enzymes which regulate mobilisation and deposition of fatty acids in adipose tissue in a reciprocal manner.[454] Reduced insulin action leads to increased lipolysis in adipocytes with increased FFA release, which can mediate many adverse metabolic effects, most notably further insulin resistance [446] and dyslipidaemia.[158] In diabetes greater amounts of fatty acids returning to the liver are reassembled into TGs and secreted in VLDL.[455] In the presence of increased concentrations of VLDL in circulation, CETP will exchange VLDL TGs for cholesteryl ester in the core of HDL, which results in smaller HDL particles, which are more rapidly cleared from plasma.[456] This explains the hypertriglyceridemia and reduced HDL commonly observed in Type 2 diabetes.[158] Cholesteryl ester transfer protein also exchanges VLDL triglyceride for cholesteryl ester in the core of LDL and the net effect is a decrease in the size and an increase in the density of LDL particles.[158] *In vitro*, small dense LDL can be oxidised more easily, and it binds to LDL receptors less avidly, than normal LDL. As a result diabetic patients are at an increased risk of cardiovascular disease. Many clinicians measure LDL density and/or size to predict

risk of cardiovascular disease. An increase in waist circumference (WC) and waist-height ratio (WHpR) has been found to be associated with small dense LDL particles.[158, 457] Therefore, intra-abdominal fat is a critical determinant of an atherogenic lipoprotein profile, including disruptions in the distribution of cholesterol in the different lipoprotein fractions.[443]

The characteristic lipid profile in an individual with Type 2 diabetes (increased plasma VLDL, increased TGs, decreased plasma HDL and less commonly, an increase in LDL-cholesterol),[158] may have important implications for MP levels in the eye, as studies have shown that the carotenoids lutein and zeaxanthin are primarily transported by HDL in plasma.[18, 19] High density lipoprotein is a known ligand for SR-B1, and this suggests a "piggy back" mechanism of lutein and zeaxanthin uptake into the retina via RPE cells which is mediated by SR-B1.[326] This hypothesis is supported by evidence from lutein deficiencies observed in the retina of chickens with a genetic HDL defect but not in other organs, implying a major role for HDL in the receptor-mediated transport of lutein to the eye.[18] It has been argued that the subspecies of HDL containing apolipoprotein E supplies lipids and lipid soluble lutein and zeaxanthin to the retina, and that by increasing HDL, retinal lutein levels may be simultaneously augmented.[441]

Omega-3 long chain-PUFA supplementation may also lead to increases in plasma HDL.[458] Recent epidemiological studies indicate that increased dietary long chain-PUFA intakes enhance MPOD,[459] and that DHA facilitates the accumulation of lutein in the blood and macula. The mechanism by which DHA increases MPOD may relate to its effect on the transport and uptake of lutein into the macula by mediating changes

in lipoprotein levels.[460] Putatively, this effect could also be mediated by the anti-inflammatory effects of DHA, but this is conjectural and no direct evidence supports this effect within the retina.

One observational study found that the correlation between MPOD, plasma lutein, zeaxanthin and lipids differed depending on the age profile of participants.[461] In younger adults, MPOD was found to be influenced by plasma lutein, whereas in older adults, circulating lipids were an added determining factor.[461] Mares *et al*.,[384] queried whether any association with plasma lipids is truly strong enough to play a meaningful role in the pathogenesis of MPOD depletion. One study which used a diabetic cohort, involving a small group of 43 participants (14 controls, 17 with diabetes but without retinopathy and 12 with diabetes and with mild non-proliferative retinopathy), found no significant correlation between MPOD and plasma lipid levels.[6] Our own research group, however,[7] reported lower levels of MPOD in Type 2 versus Type 1 diabetes and noted significantly lower HDL values within the Type 2 group, with raising the possibility that lower HDL levels may have contributed to this difference. More recently, the DiVFuSS study [21] has demonstrated significant improvements in plasma LDL cholesterol level (9% mean decrease in the intervention group vs 1% mean increase in placebo group); HDL cholesterol (7% mean increase in the intervention group vs 3% decrease in placebo group); and TGs (8.6% mean decrease in the intervention group vs 2% mean increase in placebo group), suggesting a positive effect of the study formulation on plasma lipids. Whilst this effect was likely attributable to other non-carotenoid components of the supplement, the synchronous increase in MPOD observed in the intervention group may have been partially mediated by these favourable changes in lipid profile and their consequential impact on carotenoid

transport to the retina.[18, 19, 71] In the broader context, the evidence from this study is sufficiently robust to prompt further lines of investigation into the role of MP supplementation either in isolation or as a multi-component supplement in diabetes. The research to date, however, is not conclusive and it remains unclear from the limited amount of observational and RCT data, whether plasma lipids (primarily HDL) play a meaningful role in determining MPOD status. Given the theoretical basis for an association between lipid profile and MP status, and the fact that lipid profile is often altered deleteriously in diabetes, further investigation is warranted. The relationship between dyslipidaemia and lower levels of MP is outlined in Table 6.4.

Table 6.4: Summary of studies linking MPOD with plasma lipids.

Author & Year	Design	Sample	Results
Mares et al (2006).[384]	Cross-sectional study on women. MPOD measured by HFP.	Sub sample of women who participated in the CAREDS (N = 1698).	Plasma lutein & zeaxanthin correlated with plasma cholesterol (r = 0.17) but adjustment for this did not affect the direct association between plasma levels and MPOD.
Lima et al (2010).[6]	Cross-sectional study with 3 test groups: non-diabetic controls, diabetic participants without retinopathy & diabetic participants with retinopathy.	43 Type 2 diabetes participants with and without retinopathy matched to controls.	No significant associations were observed with lipid levels and MP (p = 0.08).
Loane et al (2010).[71]	Cross-sectional study on healthy men & women, without ocular disease, aged between 20 -70 years.	Total no of subjects: N=302. N= 181 with negative family history of AMD; N=121 with positive family history of AMD.	A significant and positive association between plasma HDL and plasma concentrations of lutein. A significant inverse association between plasma TG concentration and both plasma lutein concentration and MPOD in those with a positive family history of AMD.
Renzi et al (2012).[462]	Cross-sectional study on young healthy subjects. MPOD measured by HFP.	Relations between plasma lutein & zeaxanthin, MPOD and lipoprotein levels (N=108).	HDL were significantly ($p < 0.05$) related to MPOD (r = 0.33), to plasma lutein (r = 0.36) and to plasma zeaxanthin (r = 0.26). MPOD was also significantly related to TC (r = 0.19).

Olmedilla-Alonso et al (2014).[461]	Cross-sectional study assessing plasma concentrations and intake of lutein and zeaxanthin and MPOD using HFP.	Healthy men and women aged (20-35 and 45 -65 years) (N = 108).	MP only correlated with plasma lutein & zeaxanthin when expressed in terms of cholesterol + TGs (p = 0.012) or LDL (p = 0.017). In the older group relationships were also seen with LDL (p = 0.002) and HDL (p = 0.004).
Nagai et al (2015).[463]	Cross-sectional study assessing plasma concentrations of carotenoids and lipoproteins. MPOD was measured using HFP.	55 healthy volunteers (110 eyes) aged 23-53 years.	MPOD was positively correlated with plasma concentrations of lutein (p=0.00001), zeaxanthin (p=0.0005) and dietary lutein intake (p=0.002). Plasma oxidised LDL was inversely correlated with MPOD (p = 0.006), after adjusting for age, sex, and plasma lutein.
Chous et al (2015).[21]	DiVFuSS RCT: Type 1 and Type 2 diabetes with no diabetic retinopathy or mild to moderate non-proliferative diabetic retinopathy versus placebo. Intervention group received 2 daily doses of a xanthophyll, antioxidant and plant extract supplement.	Total group (N=67); Type 1 (N=27); Type 2 (N=40). Intervention DiVFuSS supplement (N=39); Type 1 (N=16); Type 2 (N=23). Placebo (N=28); Type 1 (N=11); Type 2 (N=17).	Significant improvement in MPOD (p values: 0.008 to <0.0001) and visual function on all measures (p values: 0.008 to <0.0001); Mean LDL, HDL and TG levels within the intervention group at 6 months were -9%, +7%, -8.6% respectively versus +1%, -3%, -2% respectively in the placebo group.

Abbreviations: MPOD, macular pigment optical density; HFP, heterochromatic flicker photometry; CAREDS, carotenoids in age related eye disease study; MP, macular pigment;

AMD, age-related macular degeneration; TG, triglyceride; TC, total cholesterol; HDL, high density lipoprotein; LDL, low density lipoprotein; DiVFuSS, the diabetic visual and function supplementation study; RCT, randomised control trial.

6.5 Causal mechanisms and metabolic perturbations associated with lower macular pigment in diabetes

The candidate causal mechanisms explored herein as contributors to lower MPOD levels in diabetes relate primarily to Type 2 diabetes (or poorly controlled Type 1). The complex inter-relationship between oxidative stress/inflammation and the metabolic correlates of diabetes (adiposity, dyslipidaemia, insulin resistance, hyperinsulinemia and hyperglycaemia); and their associations with MPOD, both individually and as a whole, represent a significant scientific challenge. In summary, our analysis indicates that MPOD might be depleted through at least four causal mechanisms previously described, all of which are outlined in Figure 6.2.

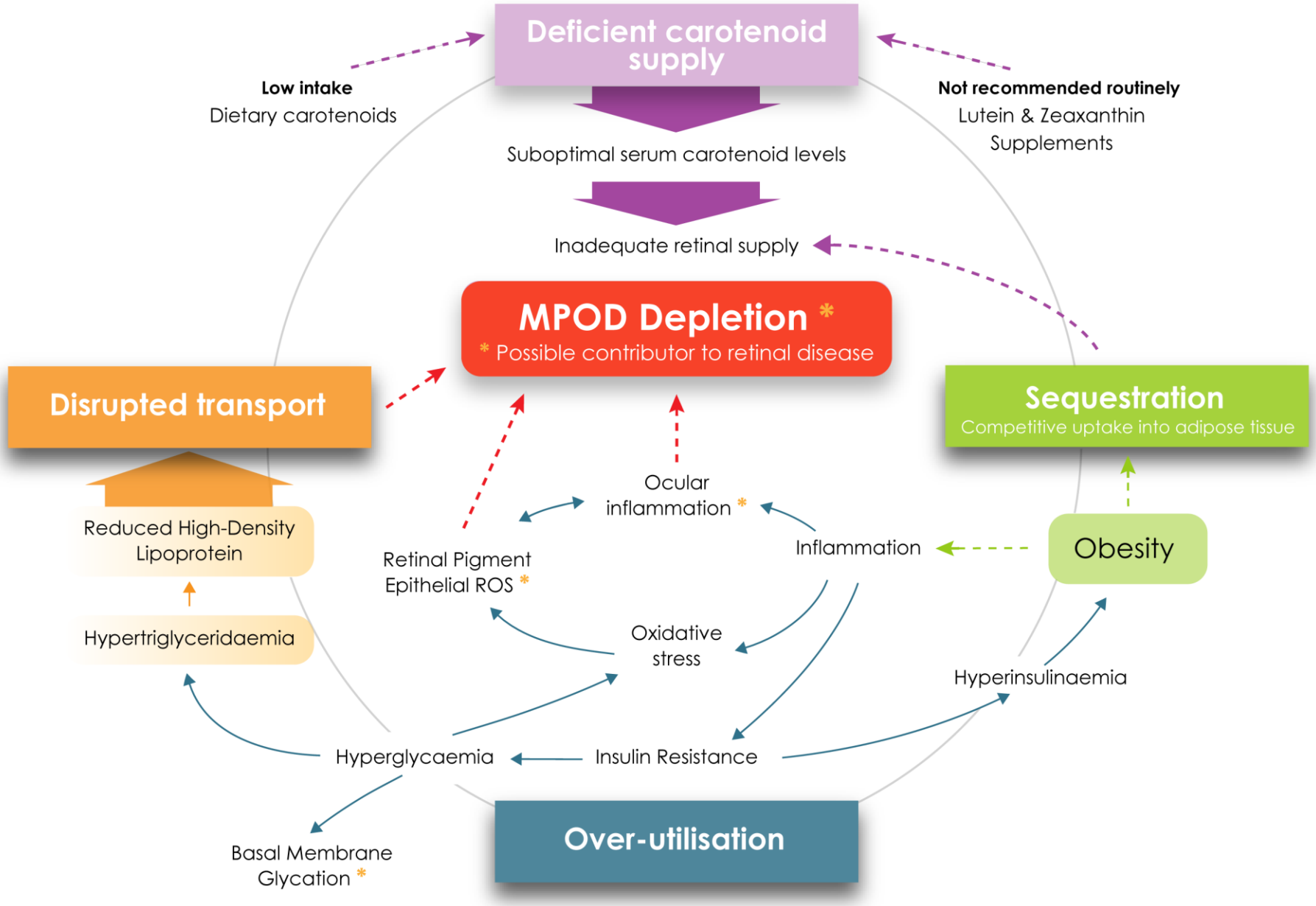

Deficient carotenoid supply
Low intake
Dietary carotenoids
Not recommended routinely
Lutein & Zeaxanthin
Supplements
Suboptimal serum carotenoid levels
Inadequate retinal supply
MPOD Depletion *
* Possible contributor to retinal disease
Disrupted transport
Sequestration
Competitive uptake into adipose tissue
Reduced High-Density Lipoprotein
Hypertriglyceridaemia
Ocular inflammation *
Retinal Pigment Epithelial ROS *
Inflammation
Obesity
Oxidative stress
Hyperinsulinaemia
Hyperglycaemia
Insulin Resistance
Basal Membrane Glycation *
Over-utilisation

Figure 6.2: Metabolic perturbations and supply deficiencies contributing to MP depletion in diabetes.

Abbreviations: MPOD, macular pigment optical density; ROS, reactive oxygen species.

6.5.1 Dietary determinants of MPOD in diabetes

First and foremost, patients with Type 2 diabetes may experience lower levels of MP as a result of poor dietary intake of antioxidant nutrients (lutein, zeaxanthin and *meso*-zeaxanthin). Lutein and zeaxanthin are not synthesised *de novo* in humans, therefore, a diet rich in fruit and vegetables is necessary to enhance MP levels in the eye.[22] While the exact cause of diabetes is still not fully understood, dietary factors have garnered particular attention over the last number of years. In fact, some research indicates that up to 74% of Type 2 diabetes cases are directly attributable to obesity.[464]

Many studies have reported a positive association between a high intake of sugars and the development of Type 2 diabetes.[465-467] Research has also found an association between high fat intakes and Type 2 diabetes and impaired glucose tolerance,[468, 469] with some literature suggesting that high-fat, low-carbohydrate diets are associated with the onset of Type 2 diabetes.[468] A global change in dietary intake has occurred over recent decades resulting from an increased use of sweeteners such as fructose and sucrose by the food industry.[470] Recent evidence suggests a causal link between the high intake of sugar-sweetened soft drinks and obesity and diabetes. This is thought to arise from the large amount of fructose in these soft drinks, which impairs insulin sensitivity and raises blood glucose levels and BMI to dangerous levels.[466, 471] Fructose consumption increases postprandial TG concentrations within hours,[472] which

suggests that postprandial hypertriglyceridaemia is one of the earliest metabolic perturbations associated with fructose consumption. The most likely mechanism for this hypertriglyceridaemia is increased hepatic *de-novo* lipogenesis, which in turn upregulates VLDL production and secretion,[473] factors which have a knock on suppressive effect on HDL, a known transporter of carotenoids to the eye.[18, 19]

Studies have also found an inverse correlation between the intake of green leafy vegetables and risk of developing Type 2 diabetes; [214, 474] and that the consumption of fruits and vegetables may protect against the development of Type 2 diabetes, as they are rich in micronutrients, fibre and antioxidants,[474] dietary components also known to enhance MPOD. A recent meta-analysis revealed that a higher intake of fruit, especially berries and green leafy vegetables, yellow vegetables and cruciferous vegetables or their fibre, is associated with a lower risk of Type 2 diabetes.[214] Health benefits have also been observed with the Mediterranean diet,[475, 476] a diet which contains an abundance of fruit and vegetables. While, the beneficial effects of fruits and vegetables on diabetes risk are likely to be multiple, these foods are the primary dietary source of carotenoids including lutein and zeaxanthin, highlighting their consumption as a key determinant of MPOD. Lower levels of MPOD in diabetes, might also be associated with high intakes of fried foods and fat,[477] alcohol,[478] red and processed meat,[479] sugar-sweetened beverages,[465, 466] and high glycaemic index foods,[480] all of which have been linked with higher levels of visceral fat and increased WC. Numerous studies have shown that obesity (in particular, central obesity) can increase the risk of Type 2 diabetes.[481]

While the body's natural defence against oxidative damage and inflammation is

neutralisation by endogenous antioxidants (enzymatic & non enzymatic), in association with exogenous antioxidants (vitamin C, vitamin E, carotenoids, β-carotene, lutein, zeaxanthin and *meso*-zeaxanthin),[177, 419] these antioxidant defences may be compromised in diabetes. Hence, it is clear that both over utilisation and under supply of antioxidant nutrients may contribute to lower levels of MP in the diabetic retina.

Based on the outlined associations between nutritional and dietary factors and the components of the metabolic syndrome which may contribute to MPOD depletion, we outline dietary factors which: 1) may exacerbate the metabolic correlates associated with Type 2 diabetes (Table 6.5 a) and 2) may help to prevent or resolve the metabolic perturbations associated with Type 2 diabetes (Table 6.5 b).

Overall, western dietary habits are a significant factor in the development of the metabolic syndrome. The continuous over-provision of energy via dietary carbohydrate and lipid, when unmatched by physical activity-induced energy expenditure, leads to a state of excess adiposity, chronic low grade inflammation, and oxidative stress and dyslipidaemia; features associated with Type 2 diabetes and which may lead to MPOD depletion.

The importance of diet cannot be overemphasised in diabetes management. Given the variety of mechanisms which may contribute to MPOD depletion and the broader ocular risks associated with such MPOD depletion in diabetes, a comprehensive dietary approach is therefore necessary to optimise ocular health and MPOD status. The generous provision of brightly coloured fruits and vegetables which are rich in a

broad range of antioxidants including the target carotenoids lutein, zeaxanthin and *meso*-zeaxanthin represents an obvious recommendation. This, however, should be complimented by the inclusion of wholegrain fortified breakfast cereals for their folate, vitamin D and anti-obesogenic fibre content; and with plenty of oily fish to provide further vitamin D and omega-3 essential fatty acids to attenuate inflammation and correct or prevent hypertriglyceridaemia. These foods should be used to displace those which are high in fat, saturated fat, refined sugar and fructose; for example oily fish should replace red or processed meats at several main meals each week, fruit or raw vegetables should be used to replace snack foods high in fat, saturated fat, *trans*-fat and refined sugar (e.g. baked goods and confectionery); and low fat fortified milk should replace sugar-sweetened soft drinks. Starchy carbohydrates should be evenly distributed over the day to achieve better glycaemic control, with sterol or stanol enriched products used to reduce plasma LDL and consequently enhance LDL: HDL ratio. Finally, from a broader lifestyle perspective, these individuals should be advised to moderate their alcohol consumption to no more than two standard drinks per day with several alcohol-free days each week, and to engage in moderate intensity exercise (e.g. walking) on most days of the week to enhance their insulin sensitivity and optimise their blood glucose and lipoprotein profiles. Table 6.5 a. outlines the dietary factors causally associated with the metabolic correlates of Type 2 diabetes, and Table 6.5 b. outlines the dietary factors associated with protection against the metabolic perturbations of Type 2 diabetes.

Table 6.5: Dietary factors associated with the metabolic correlates of Type 2 diabetes.

Metabolic correlates of Type 2 diabetes.	a. Causal dietary factors associated with the metabolic correlates of Type 2 diabetes.	b. Preventative dietary factors associated with the metabolic correlates of Type 2 diabetes.
Oxidative Stress	High protein (red meat),[479] high fat (saturated fat).[482, 483] High carbohydrate (refined sugar, fructose).[465, 466, 484]	Whole grains (fibre),[485] nuts (fibre, omega-3 PUFA).[486] Fruit and vegetables (antioxidants vitamin C & E, β carotene, lycopene, quercetin, resveratrol, flavonoids, lutein, zeaxanthin & *meso*-zeaxanthin).[5, 15, 21, 22, 214, 250, 259, 474, 476, 487, 488] Fish (omega-3 PUFA),[489] legumes (protein, phytochemicals).[490]
Inflammation	High fat (saturated fat, *trans* fat).[491-493] High carbohydrate (refined sugar, fructose).[484, 494, 495] Alcohol.[496]	Oily fish and omega-3 FAs,[489] folate,[497] vitamin D.[498] Fruit & Vegetables (antioxidants vitamin C & E, β carotene, lycopene, quercetin, resveratrol, flavonoids, lutein, zeaxanthin & *meso*-zeaxanthin).[377, 487]
Obesity	Excessive energy consumption.[499] High fat (saturated).[500-503] High carbohydrate (refined sugar, fructose).[465, 466]	Diet & exercise,[504] high dietary fibre.[505] High fruit and vegetable (antioxidants vitamin C & E, β carotene, lycopene, quercetin, resveratrol, flavonoids, lutein, zeaxanthin & *meso*-zeaxanthin),[487] high milk intake.[506]

Dyslipidaemia	High fat (saturated fat, trans fat),[507-509] high carbohydrate (refined sugar, fructose),[510, 511] Sodium,[510] alcohol (especially binges),[512] low physical activity.[513]	High dietary fibre,[505] omega-3 FAs,[514, 515] plant stanols/sterols,[516] physical activity.[458, 513]

Abbreviations: PUFA, polyunsaturated fatty acid; FA, fatty acid.

6.6 Limitations

It is important to note the significant limitations which currently prevail in the reviewed literature. The heterogeneity of studies is a major drawback. Small sample sizes, the merging of Type 1 and Type 2 diabetes patients in statistical analyses, and the complex interplay of diabetes and its accessory factors (adiposity, dyslipidaemia, oxidative stress and inflammation) with MPOD status are also challenging. While there is some preliminary positive evidence, primarily from animal studies [14, 20] and the DiVFuSS trial,[21] that higher MP levels may be protective against retinal damage, there is limited evidence that low MPOD levels are detrimental to ocular health in diabetes, and the actual benefits of higher MPOD in terms of retinal disease development and/or progression in diabetes remain to be elucidated.

6.7 Conclusion

This review outlines our present understanding of the relationship between diabetes and MPOD. Macular pigment appears to be generally lower in diabetic populations compared to healthy controls,[6] and this is noted particularly amongst patients with Type 2 diabetes.[7] Impaired defence against ROS at the retina may not only be attributable to lower intake of foods rich in carotenoids and other antioxidants (e.g. fruit, vegetables, legumes) however, but may also arise from the increased utilisation of these antioxidants in diabetes (via chronic inflammation and oxidative stress). Adiposity, particularly abdominal fatness, is closely linked to Type 2 diabetes, and is a significant factor in the condition's pathogenesis. Excess fat tissue may not only compete with the retina for uptake of dietary carotenoids, but may also contribute to increased oxidative stress and inflammation, factors which may

negatively impact MP levels in the eye. Similarly, the hyperglycaemia which is characteristic of diabetes, also contributes to inflammation and oxidative stress, and their depletive effects on MPOD. Dyslipidaemia, more common in poorly controlled diabetes, is a metabolic correlate which may adversely affect the transport and assimilation of carotenoids into retinal tissue, by limiting the availability of their principal carrier HDL. The chronic low-grade inflammation and increased oxidative stress observed in patients with Type 2 diabetes may also increase the use of these carotenoids at the molecular level, and in so doing, may elevate our dietary requirement for them.

In view of the limited evidence available, supplementation with lutein, zeaxanthin, and/or *meso*-zeaxanthin, either with or without other antioxidants, represents a fertile area for future interventional research. It is important to recognise, however, that in order to minimise the risk of MPOD depletion and/or ocular damage in diabetes, it is not sufficient to simply supplement with macular carotenoids. Rather, our review suggests the need for a more holistic evaluation of diet and lifestyle in relation to MPOD in diabetes. In this context, inflammation and oxidative stress, the dual pathogenic driving forces of diabetes which occur independent of hyperglycaemia, may be amenable to dietary intervention and nutritional supplementation. The incorporation of a number of dietary modifications (e.g. enhanced antioxidant, vitamin D and omega-3 fatty acid intake; and reduced fat, saturated fat, *trans*- fat and refined sugar consumption); alongside lifestyle changes such as increased physical activity; may not only address the under-supply of macular carotenoids, but may also abrogate the competitive storage, transport, inflammatory and oxidative features of Type 2 diabetes, which may compromise

MPOD status. The safety and low cost of such interventions further commend their potential value as a therapeutic adjunct in diabetic eye disease, and may come to represent an essential element of ocular care for this important and expanding patient group.

7. IDENTIFICATION OF SURROGATE BIOMARKERS FOR THE PREDICTION OF PATIENTS AT RISK OF LOW MACULAR PIGMENT IN TYPE 2 DIABETES.

7.1 Abstract

Purpose

This cross-sectional study compared MP levels among persons with Type 2 diabetes relative to healthy controls. Additionally, a range of behavioural, anthropometric, clinical and plasma measures were explored as possible predictors of low MPOD in diabetes.

Methods

Two health status groups; Group 1: Type 2 diabetes (n=188), and Group 2: Healthy controls (n=2,594) completed a full MP assessment using c-HFP, as part of TILDA. Clinical [blood pressure; cataract status; MPOD] and anthropometric [waist (cm); weight (kg); hip (cm)] measurements were taken, and a blood sample drawn for analysis of plasma biomarkers [lipoproteins; inflammatory markers (CRP and vitamin-D)].

Results

One-way analysis of variance revealed lower MPOD in subjects with Type 2 diabetes relative to controls (p=0.047). Amongst participants with diabetes, those with low plasma vitamin D (≤50 nmol/L) had significantly lower mean MPOD compared to those with sufficient plasma vitamin D levels (>50 nmol/L) (0.173(0.148) vs. 0.226(0.145); p=0.006). Concomitantly, MP was significantly lower in diabetes participants with raised plasma TG over HDL lipoprotein ratio

(TG/HDL); values >1.74 mmol/L (0.172 (0.140) vs 0.215 (0.152); p=0.039). Macular pigment was also significantly lower in diabetes participants with hypertension (>140/90 mmHg); (0.177(0.130) vs 0.228(0.166); p=0.043). Body mass index, WHtR, and WC were all significantly negatively correlated with MPOD (Pearson's correlation, p<0.05 for all). Significant correlates of MPOD in the multivariate regression model included smoking, cataract, and vitamin D, which collectively contributed 18.5% of the overall variability in MPOD status amongst participants with Type 2 diabetes.

Conclusions

This study provides additional evidence that low MP may indeed be a feature of Type 2 diabetes and further identifies smoking, cataract, and vitamin D status as plausible predictors of low MPOD amongst persons with Type 2 diabetes.

7.2 Introduction

The prevalence of diabetes has been increasing steadily over recent decades and is now reaching epidemic proportions.[517] Type 1 and Type 2 diabetes are the two main forms of diabetes, however, Type 2 diabetes is much more common and is estimated to affect more than 500 million people worldwide.[518] Diabetic retinopathy, a debilitating microvascular complication of diabetes, is the most common cause of vision loss in people with diabetes and is a leading global cause of vision impairment and blindness among working-age adults.[26]

The pathogenesis of diabetic retinopathy is multifactorial and remains poorly understood. Chronic hyperglycaemia induces oxidative stress in the retina,[355] and it is thought that hyperglycaemia causes tissue damage through a number of major pathways, including the polyol pathway, activation of PKC, upregulation of AGE product formation and activity of the hexosamine pathway.[412] Interaction of these biochemical pathways may cause a cascade of events, such as apoptosis, oxidative stress, inflammation, and angiogenesis, which can lead to damage of the diabetic retina, as reviewed by Al-Kharashi.[519] It is thought that hyperglycaemia leads to a dysregulation of inflammation, which in turn leads to an increase in the level of pro-inflammatory proteins.[372]

Inflammatory processes underlie many of the functional retinal vasculature alterations observed histologically in early diabetic retinopathy.[194] Animal and human studies have confirmed that all retinal cell types, including inner retinal neurons, Muller cells, and astrocytes, are damaged by diabetes.[204] Recognising both the clinical and histological aspects of retinal change in diabetic macular oedema

and diabetic retinopathy is essential to understanding the mechanisms of vision loss in diabetes, and in developing early-stage clinical interventions that effectively target the specific aetiologies and underlying pathological mechanisms of the condition. In fact, insulin resistance, impaired glucose tolerance, and Type 2 diabetes may exist for many years before clinical retinal signs become evident.[520]

The body's natural defence against oxidative damage and inflammation is the neutralisation of ROS with endogenous antioxidants, both enzymatic and non-enzymatic.[401] These endogenous antioxidants work with exogenous antioxidants (vitamin C, vitamin E, and carotenoids such as β-carotene, lutein, zeaxanthin, and *meso*-zeaxanthin),[177] together balancing redox status. The carotenoids, lutein, zeaxanthin, and *meso*-zeaxanthin, collectively known in the eye as MP; confer potent antioxidant and anti-inflammatory effects at the macula.[5,283] These carotenoids are uniquely concentrated in the inner and central layers of the primate macula, and while there have been numerous studies looking at the role of carotenoids and other nutrients in the prevention of AMD, reviewed elsewhere,[226] the association between diabetes and MPOD levels, however, has received somewhat less attention.

The evidence that does exist, however, suggests that the relationship is worth exploring in more detail. Plasma concentrations of lutein and zeaxanthin have been observed to be significantly lower in patients with diabetic retinopathy.[15] Furthermore, MP has been found to be significantly lower in patients with diabetes,[6] with one study reporting lower levels of MP in Type 2 versus Type 1 patients.[7] It is also possible that MP may confer therapeutic benefits in diabetic eye disease. A

number of studies have explored the effect of MP supplementation in diabetes. Although the evidence is far from definitive, improvements in structural and functional measures of ocular health in response to macular carotenoid supplementation have been found. The earliest of these studies reported improvements in vision and macular oedema following supplementation.[15] The DiVFuSS study suggested that a nutritional supplement containing lutein and zeaxanthin mitigated the damaging effects of systemic inflammation on ocular function and that these beneficial effects may have been mediated by enhancements in MPOD.[21] Improvements in visual function have also been observed in patients with diabetes following supplementation, including in contrast sensitivity [521] and in electrophysiological indices of retinal function.[387]

The reasons why MPOD levels might be adversely affected in diabetes are yet to be elucidated, but a number of factors might be important. At presentation, Type 2 diabetes is most often accompanied by other co-morbidities including overweight/obesity, insulin resistance, hypertension and dyslipidaemia,[522] which may adversely affect MP by compromising the availability,[54] transport,[18] assimilation,[18] and maintenance/retention of dietary carotenoids in the retina. *De novo* synthesis of carotenoids is not possible in humans, therefore, the chronic low-grade inflammation,[372] and pro-oxidative environment,[355] associated with Type 2 diabetes may negatively impact MPOD levels in the eye.

Macular pigment levels can be measured *in vivo*, but are not routinely measured in clinical practice. Given the possibility that higher levels of MP may be beneficial for vision and ocular health in diabetes, the investigation of surrogate indicators of

MP status that are more readily and routinely measured is merited. The capacity of such an alternate biomarker to expedite the identification and treatment of patients at risk of low MP could be particularly important given that retinal damage can occur long before visual signs of diabetic retinopathy are evident. This study was designed, therefore, to compare MPOD in participants with and without Type 2 diabetes and, more importantly, to explore a range of behavioural, anthropometric, clinical and plasma biomarkers as possible predictors of risk for low MPOD among individuals with Type 2 diabetes.

7.3 Methods

Study population

The Irish Longitudinal Study on Ageing is a large prospective cohort study examining the social, economic and health factors which influence healthy ageing in older adults resident in Ireland.[523] Cross-sectional data from TILDA was analysed in this study. A stratified clustered sample of 8,175 individuals, representative of the population of Ireland, aged 50 years and over, participated in Wave 1 of this study, which took place between October 2009 and July 2011. The study design of TILDA has been described in detail elsewhere.[523, 524] Health and lifestyle data were captured in participants' own homes using computerised aided personal interview (CAPI).[525, 526] The presence or absence of eye pathology was determined using the CAPI with the question ''has a doctor ever told you if you had any of the following conditions: diabetes, AMD, cataracts or glaucoma''. Participants were also asked whether they had ever been told by a doctor if they had high cholesterol or high blood pressure. Participants were asked to record all medications that they took on a regular basis, including those related to diabetes,

such as oral hypoglycaemic agents and/or insulin, which were coded using the Anatomic Therapeutic Classification (ATC) system. Participants were asked if they currently smoked or had ever smoked cigarettes regularly (daily for at least a year), and were categorised as never smoked, past smokers or current smokers. The International Physical Activity Questionnaire was used to classify participants' level of physical activity into low, moderate or high levels. All participants were invited to attend a health assessment in one of two locations, Dublin or Cork. Macular pigment measurement was only conducted on participants who attended a health centre, therefore, a number of participants were automatically excluded from the current analysis: *i.e.* if they were unable to travel to a health centre or opted for a home assessment instead (n=875) and/or refrained from having either a home or health assessment (n=2,025). A total of 5,275 individuals participated in a health assessment, carried out by trained research nurses (Figure 7.1). This research adhered to the tenets of the Declaration of Helsinki and was approved by the Technological University Dublin Research Ethics Committee. All participants provided written informed consent prior to participation in the study.

Diabetes classification

Diabetes type was classified into 'no diabetes', 'pre-diabetes', 'diagnosed diabetes' and 'undiagnosed diabetes'. Diagnosed diabetes was identified from the CAPI with the question 'has a doctor ever told you that you have diabetes or high blood sugar?' and also from prescribed diabetes medications at the time of the interview, identified using the ATC codes 'A10A' for insulin and 'A10B' for oral hypoglycaemic medications. A blood sample was provided for plasma analysis. Glycated haemoglobin, a long-term indicator of glycaemic control, was measured and participants were further classified as having 'pre-diabetes,' and 'undiagnosed

diabetes' against American Diabetes Association cut-off values.[24] The TILDA protocol for blood sample collection, processing and storage has been described previously.[525] Eleven respondents who reported a doctor's diagnosis of diabetes before the age of 40 and who were on insulin therapy at the time of interview were excluded from analysis due to the suspicion that they might have Type 1 diabetes, in line with previously published data [130] (Figure 7.1). Duration of disease was quantified by asking those with a previous diagnosis 'how old they were when first diagnosed?'

Macular pigment optical density assessment

Corrected VA was measured in both eyes using the ETDRS LogMAR chart at a distance of 4 metres, using the participant's existing prescription where necessary. The eye with the best VA was chosen for the MP assessment or if there was equal vision in both eyes, the right eye was chosen. The MPOD was measured by HFP centrally at 0.5° of retinal eccentricity using the Macular Metrics Densitometer (Macular Metrics, Rehoboth, MA). This device was modified specifically for the TILDA study from a device originally described by Wooten et al.[334] The established method employed for measuring MP has been described in detail previously.[56] In brief, the subject viewed a stimulus that alternated between a wavelength which is absorbed by MP and one that is not. To account for the fact that participants may have different temporal (*i.e.* flicker) rates, the HFP task was customised for each subject, according to their age.[335] If a fixed flicker frequency was used instead then a subject with a low flicker sensitivity (*i.e.* low critical flicker fusion frequency) would experience a large null flicker zone. While this does not prevent the test from being completed, it could lead to an over or underestimation of MPOD. Conversely, a subject with a high critical flicker fusion frequency may not be able to eliminate

flicker and this would make the test more difficult to complete. A 'bracketing' protocol was adapted in the TILDA study to allow quick but accurate customised MPOD measurements to be obtained, both centrally at 5^0 and peripherally at 7^0.[56] The examiner pre-set the radiance dial, either at the lowest blue light intensity (ascending measurement) or the highest blue light intensity (descending measurement), and a total of 6 measurements were taken. In other words, the examiner pushed a button that alternated the blue/green ratio until the subject reported no flicker or that flicker had started. The radiance value obtained was recorded for the 6 measurements in the MPOD log form (central measurement where MP peaks). The examiner then selected the target and fixation point required to measure MPOD at 7° retinal eccentricity (parafoveal target at 7°), where MP is optically undetectable, and another 6 measurements were taken. All radiance values obtained (12 in total) were used to calculate MPOD by taking the log ratio of MP peripherally (7^0) from the log ratio of MP centrally (0.5^0) and in this way the MPOD value at 0.5° was generated for that subject. Of the 5,275 participants who attended a health assessment, 2,782 were deemed suitable for the current analysis and successfully completed an MPOD assessment (participants with diabetes, n=188; non-diabetic controls, n=2,594). A number of subjects were excluded from the overall MPOD analysis (n=2,493) and possible reasons for this included: poor fixation, technical issues, poor VA (VA 6/18 Snellen (≤ 0.5 LogMAR)) and retinal pathology (AMD, glaucoma). Because Type 1 and 2 diabetes are considered different clinical conditions, the presenting features of the condition (adiposity, dyslipidaemia, oxidative stress, and inflammation) may not be uniform across both and may have different relationships with MPOD, therefore, a number of participants with suspect Type 1 diabetes (n=11), were excluded from the current

study. Participants with pre-diabetes (HbA1c: 5.7 - 6.4%) were also excluded from the current analysis (n=111). Of the 8,500 participants that took part in Wave 1 of TILDA, 188 Type 2 diabetes participants and 2,594 controls were deemed suitable for the study (Figure 7.1).

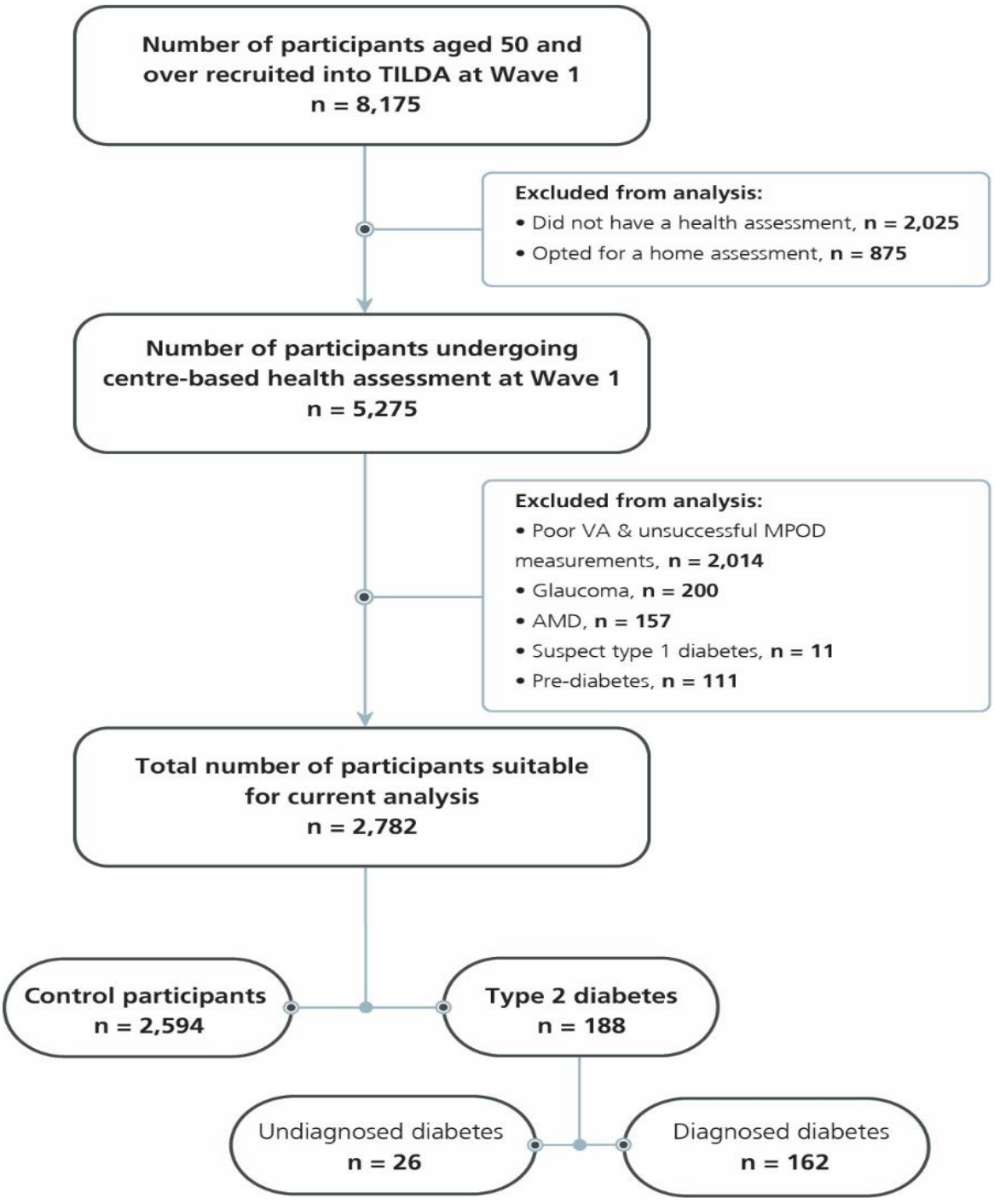


Figure 7.1: Flowchart illustrating the selection of study participants and reasons

for exclusion from the original participant group.

Abbreviations: AMD, age-related macular degeneration; MPOD, macular pigment optical density, n, number of participants; TILDA, The Irish Longitudinal Study on Ageing; VA, Visual Acuity.

Anthropometric assessment

Height (cm) and weight (kg) were measured to one decimal place as described in detail elsewhere.[525] Body mass index was calculated from measured height and weight as: weight (kg)/height (m^2). Waist circumference and hip were measured to the nearest cm. Waist-to-height ratio and WHpR were calculated based on these measured data. Cut-offs were applied to classify participants as obese or non-obese for the following variables (BMI; WC; WHtR, and WHpR).[527]

Blood pressure

Two blood pressure (BP) measurements were taken using the OMRON^{TM} digital automatic BP monitor (Model M10-IT) with arm cuff.[525] Seated mean systolic (mmHg) and mean diastolic (mmHg) BP were used for analysis. The participant was defined as hypertensive if mean seated systolic BP exceeded 140 mmHg or mean seated diastolic BP exceeded 90 mmHg.

Plasma analysis

Respondents were not asked to fast before the health assessment. Blood was extracted using defined phlebotomy protocols,[525] and analysed for a complete lipid profile, which included TC, HDL, LDL, and TGs, measured in mmol/L. Triglyceride over HDL ratio (TG/HDL), TC/HDL ratio and non-HDL cholesterol (TC minus HDL) were calculated for subsequent analyses. Cut-offs were applied

to indicate high or ideal plasma lipid levels as per 2016 European Society of Cardiology (ESC)/European Atherosclerosis Society (EAS) guidelines.[528] Research suggests that inflammation plays a role in the development of Type 2 diabetes,[10, 529] therefore, anti-inflammatory marker plasma vitamin D [(25(OH)D); nanomoles per litre (nmol/L)] and inflammatory marker CRP, [micrograms per litre (μg/l)] were also measured. Respondents were considered vitamin D deficient if plasma levels were ≤50 nmol/L, and sufficient if plasma levels were >50 nmol/L as per the Institute of Medicine (IOM) vitamin D guidelines.[530] A threshold of >3.00 μg/l for high and ≤ 3.00 μg/l as ideal was used for plasma CRP.[531]

Statistical analysis

The statistical software package SPSS for Microsoft Windows (version 23.0; IBM Corp., Armonk, NY) was used for all analyses. To account for the fact that the study response rate varied among different subgroups of the population, inverse probability weights were calculated for the main sample using the Quarterly National Household Survey (2010).[532] Participation rates for the health centre assessment also varied according to geographic location, health, education, age, and smoking, therefore, a specific "health centre weighting" was applied. A more detailed description of the weighting procedure used in TILDA is described by Barrett et al.[532]

Our data are presented as mean ± standard deviation throughout. These data were tested for normality using the Kolmogorov-Smirnov test. For group comparison between participants with diabetes and non-diabetic controls, ANOVA was used to test for differences in means for normally distributed parameters, while the Kruskal-Wallis test was used to test differences between group medians for non-normally

distributed parameters. For categorical data, cross-tabulation with Chi-square analysis was used. The distribution of MPOD was skewed, therefore, a square root transformation of the MPOD data was performed (Figure 7.2 and 7.3). The derived data were normally distributed and used as the dependent variable for subsequent statistical analyses of MPOD on the diabetes group. For ease of interpretation, mean and SD of MPOD data is presented as the non-transformed original measure for Type 2 diabetes participants. Pearson's product-moment correlation tests were performed to assess the relationship between normalised MPOD and other study variables where appropriate. Boxplots and scatterplots were used to graphically highlight statistical findings. Multiple linear regression was used to investigate the un-confounded associations between behavioural, anthropometric, clinical and plasma biomarker indicators and normalised MPOD. The level of statistical significance was set at $p<0.05$ for all analyses.

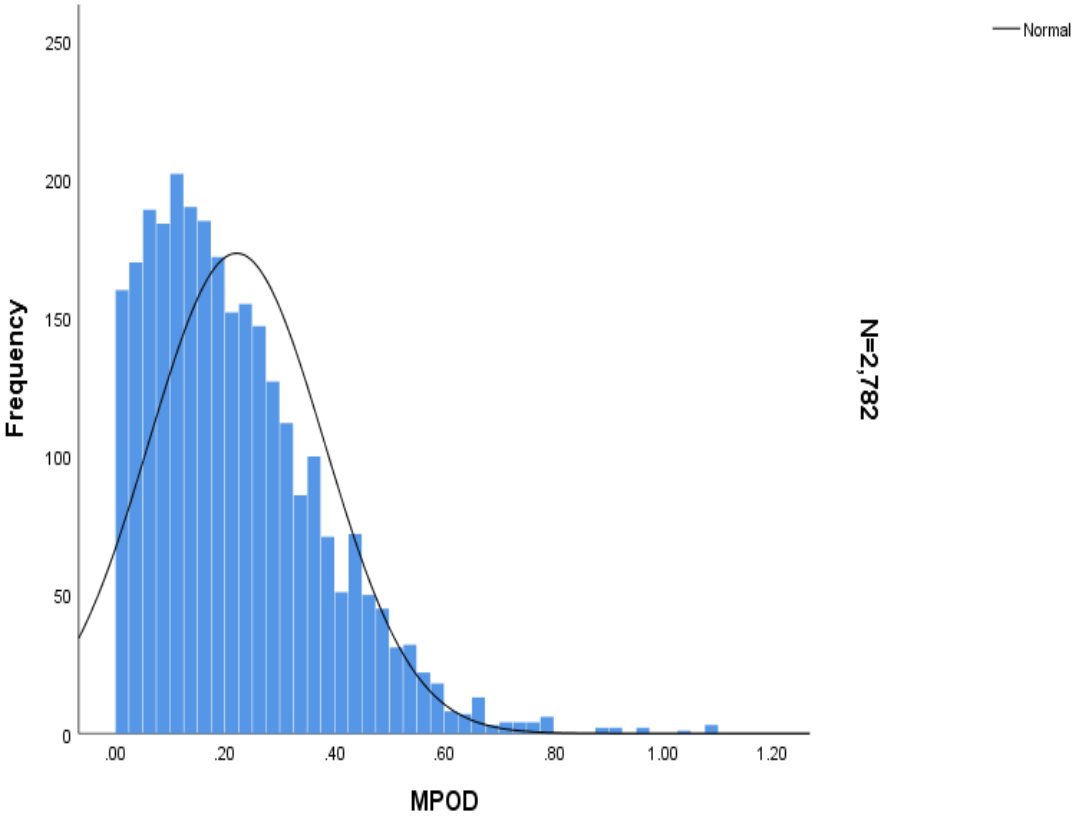


Figure 7.2: Distribution of MPOD as normal optical density value with data positively skewed [Mean =0.221; Std. Dev =0.160; N=2,782].

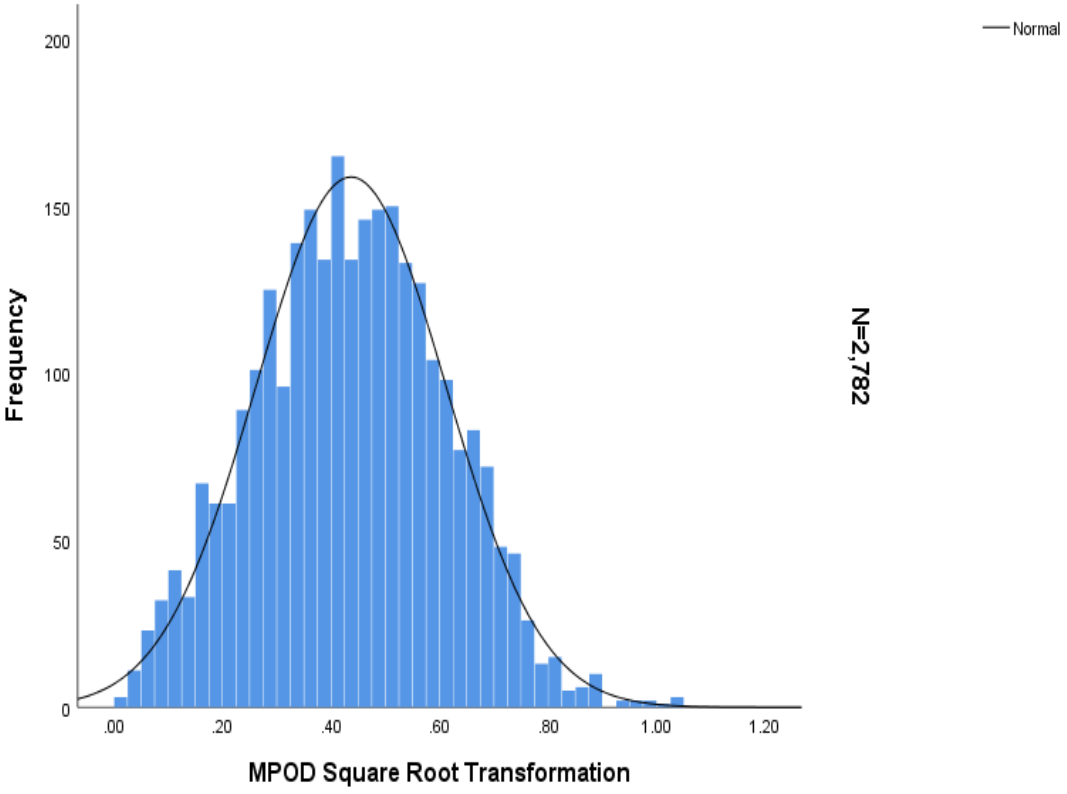


Figure 7.3: Distribution of MPOD using a square root transformation now normally distributed [Mean = 0.437; Std. Dev = 0.175; N=2,872].

7.4 Results

Two thousand seven hundred and eighty-two subjects, analysed as part of Wave 1 of TILDA, were divided into two study groups: Group 1: Non-diabetic controls (n=2,594) and Group 2: Type 2 diabetes (n=188). Respondents 'diagnosed with diabetes' and those with 'undiagnosed diabetes' were grouped together and classified as having Type 2 diabetes for subsequent analyses. Characteristics of the study population according to their diabetes status are presented in Table 7.1. Group average MPOD was 10.3% lower in participants with Type 2 diabetes [mean MPOD =0.20 (0.148)] compared with non-diabetic controls [mean MPOD = 0.223 (0.161)], and this difference was statistically significant [t (2780) = -1.989, p=0.047)]. Participants with Type 2 diabetes also differed from the control group across all other demographic, behavioural, anthropometric, clinical and plasma parameters examined (p<0.0001 for all), with the exception of education (primary, secondary or third level), alcohol intake, and plasma TC/HDL ratio (p>0.05 for these variables; Table 7.1).

Participants with diabetes were older, and more likely to be male with a current or past history of tobacco use. In general terms, the behavioural, clinical and anthropometric profile of these participants was poorer than that observed in the control group, with lower levels of physical activity, greater levels of obesity and higher prevalence of hypertension and cataract. Similarly, plasma analysis revealed that participants with diabetes were typically more hyperglycaemic, more dyslipidaemic and displayed lower anti-inflammatory marker elevation (plasma

vitamin D) and greater inflammatory marker elevation (CRP), than the control group.

Table 7.1: Characteristics of the study population according to diabetic status.

Variable	Normal Controls (n=2594)	Type 2 Diabetes (n=188)	*p*
Demographic Factors			
Age years (Mean (SD))	61.40(7.62)	64.73(8.32)	**0.0001***
Age category years † (%)			
50-64	68.12%	51.06%	**0.0001‡**
65-74	24.94%	35.64%	
>75	6.94%	13.30%	
Sex (%)			
Male	43.79%	64.89%	**0.0001‡**
Female	56.21%	35.11%	
Education (%)			
Primary	18.81%	22.34%	0.174 ‡
Secondary	41.69%	44.68%	
Third level	39.59%	32.98%	
Behavioural Factors			
Smoking (%)			
Current	13.42%	14.89%	**0.027 ‡**
Past	39.36%	47.87%	

Never	47.22%	37.23%	
Alcohol (%)			
Yes	78.83%	72.87%	0.130 ‡
No	18.47%	22.87%	
NA	6.51%	3.72%	
Exercise (%)			
Low	37.90%	54.25%	**0.0001**‡
Moderate	30.07%	28.19%	
High	32.03%	17.55%	
Anthropometric & Clinical Biomarkers			
BMI (kg/m^2)	27.98 (4.42)	32.32 (6.25)	**0.0001***
WC (cm)	93.19 (12.83)	105.47 (13.74)	**0.0001***
WHtR	0.56 (0.070)	0.63 (0.08)	**0.0001***
WHpR	0.894 (0.083)	0.96 (0.08)	**0.0001***
Hypertensive (untreated)	5.82%	2.66%	**0.0001**‡
Hypertensive (treated)	24.79%	52.19%	
Normotensive	69.39%	44.15%	
MPOD** (Mean (SD))	0.223 (0.161)	0.20 (0.148)	**0.047**††
Cataracts (%)	6.51%	13.83%	**0.0001**‡

Plasma Biomarkers			
TC (mmol/L)	5.24 (1.04)	4.27 (1.06)	**0.0001***
HDL (mmol/L)	1.59 (0.44)	1.27 (0.33)	**0.0001***
LDL (mmol/L)	3.01 (0.92)	2.24 (0.89)	**0.0001***
TG (mmol/L)	1.66(1.03)	1.94 (1.11)	**0.0001***
TC/HDL Cholesterol Ratio	3.46 (0.91)	3.473 (0.92)	0.675*
TG/HDL Cholesterol Ratio	1.19 (0.961)	1.68 (1.12)	**0.0001***
Non-HDL Cholesterol (TC-HDL) Ratio	3.65 (0.946)	2.99 (0.95)	**0.0001***
Vitamin D (nmol/L)	59.91 (25.86)	51.90 (22.72)	**0.0001***
CRP (µg/l)	2.83 (6.95)	5.23 (11.94)	**0.0001***
HbA1c (%)	5.06 (0.27)	6.22 (0.934)	**0.0001***

*‡ Chi-square test; *Kruskal–Wallis test; ††One-way analysis of variance (ANOVA);*

MPOD sqrt; P values reflect the probability associated with the given F statistic. Sig., significance.

†Age: the age division corresponds to groupings used in previously published cohort studies.[52]

***MPOD, P values are reported using MPOD square root transformation (sqrt).*

Abbreviations: BMI-body mass index; kg/m^2*- kilograms per metre squared; NA – non-applicable; WC-waist circumference; cm - centimetres; WHtR-waist-to-height ratio; WHpR-waist-to-hip ratio. HbA1c-glycated haemoglobin; TC-Total cholesterol; HDL-High-density lipoprotein; LDL-Low-density lipoprotein; TG-Triglycerides; Non HDL- Total cholesterol minus HDL; CRP-C-reactive protein; mmHg- millimetres of mercury; mmol/L -millimoles per litre; nmol/L -nanomols per litre; µg/l-micrograms per litre. Normotensive (≤140 mmHg systolic; ≤90 mmHg diastolic); Hypertensive (>140 mmHg systolic; >90 mmHg diastolic).*

Biomarker associations with MPOD among participants with diabetes

(1) Demographic and behavioural factors

Smoking was the only demographic or behavioural factor significantly associated with lower MPOD (F (2,185) = 6.019, p = 0.003). Post hoc analysis revealed that participants who never smoked had significantly higher normalised MPOD (mean=0.235(0.148)) compared with current smokers (mean=0.124(0.113); t (96); = 3.58, p=0.001). There was also a significant difference in MPOD between past (mean=0.19[0.151] and current smokers (t (116); = 2.268, p=0.025). However, there was no significant difference in MPOD between non-smokers and past smokers (t (158) = 1.723, p=0.087); [Figure 7.4 (A)].

(2) Anthropometric and clinical biomarkers

MPOD was negatively correlated with BMI, WC, and WHtR (Pearson's correlation = -0.202, -0.161, -0.189 respectively, p<0.05 for all), but not with WHpR (Pearson's r = -0.111, p>0.05). Cut-offs were applied to classify participants as obese and non-obese for all anthropometric measures, including BMI, WC, WHtR, and WHpR.[527] Macular pigment optical density, however, was not significantly different in individuals with excess compared with normal adiposity indices for all anthropometric measures (p>0.05 for all; Table 7.2). Although MPOD tended to be lower in subjects with cataracts, and in those with elevated WHtR and WHpR (all trending towards significance), only hypertension emerged in the univariate linear regression analysis as associated with lower MPOD [Table 7.2; Figure 7.4 (B)].

Table 7.2: MPOD according to anthropometric and clinical biomarkers in a diabetic population (normalised MPOD).

Variable	n (188)	Mean (SD) MPOD	25th	50th	75th	sig p
Anthropometric & Clinical Biomarkers						
BMI (kg/m^2)						
Ideal (≤30)	122	0.200 (0.148)	0.087	0.177	0.304	0.964††
Excess (>30)	66	0.199 (0.150)	0.075	0.180	0.262	
WC (cm)						
Ideal (≤102 M, ≤88 F)	55	0.224 (0.140)	0.110	0.208	0.337	0.101††
Excess (>102 M, >88 F)	132	0.191 (0.151)	0.076	0.162	0.264	
WHpR						
Ideal (≤1.00 M, ≤0.85 F)	84	0.222 (0.153)	0.090	0.210	0.305	0.075††
Excess (>1.00 M, >0.85 F)	103	0.183 (0.142)	0.072	0.138	0.259	
WHtR						
Ideal (≤0.57 M, ≤0.53 F)	45	0.237 (0.164)	0.088	0.228	0.379	0.073††
Excess (>0.57 M, >0.53 F)	142	0.189 (0.142)	0.086	0.172	0.267	
Blood pressure (mmHg)						
Normotensive (≤140/90)	83	0.228 (0.166)	0.090	0.210	0.324	**0.043**††
Hypertensive (>140/90)	105	0.177 (0.130)	0.075	0.138	0.259	

Diabetic retinopathy						
Yes	10	0.151 (0.134)	0.047	0.091	0.231	0.256††
No	152	0.204 (0.149)	0.088	0.180	0.296	
Cataracts						
Yes	26	0.150 (0.108)	0.087	0.180	0.304	0.068††
No	161	0.206 (0.153)	0.054	0.118	0.231	

†† ANOVA was used to check for MPOD differences among anthropometric and physical biomarkers. P values are reported using MPOD square root transformation (sqrt) (normalised MPOD) and reflect the probability associated with the given F statistic. Sig., significance.

The following cut-offs were applied to indicate ideal or excess obesity measures and normotensive/hypertension for the following variables: BMI-body mass index; [ideal ≤ 30; excess > 30 kg/m^2 - kilograms per metre squared; WC-waist circumference; ideal ≤ 88 F; ≤ 102 M; excess > 88 F; >102 M cm - centimetres; WHtR-waist-to-height ratio; ideal ≤ 0.53 F; ≤ 0.57 M; excess >0.57 F; >0.53 M; WHpR-waist-to-hip ratio; ideal ≤ 0.85 F; ≤1.00 M; excess > 0.85 F; >1.00 M; M=Male; F=Female; [527] Normotensive (≤140 mmHg systolic; ≤90 mmHg diastolic); Hypertensive (>140 mmHg systolic; >90 mmHg diastolic).

(3) Plasma biomarkers

Macular pigment optical density was significantly lower among participants with a raised TG/HDL ratio [Mean=0.172 (0.140)] compared to those with ideal levels (Mean =0.215(0.152); t (185); = 2.080, *p*=0.039; Table 7.3; Figure 7.4 (C)). One way analysis of variance revealed no significant difference in MPOD between those with high and low TC, HDL, LDL, TGs, non-HDL, and TC/HDL (*p*= 0.287-0.946) (see Table 7.3). Participants who were vitamin D deficient had significantly lower MPOD [mean = 0.173(0.148)] compared to participants who had sufficient levels [mean = 0.226 (0.145); t (185), = -2.796, *p*=0.006; Table 7.3; Figure 7.4 (D)]. Figure 7.5 shows a significant positive relationship between normalised MPOD and plasma vitamin D (r=0.218, *p*=0.003). There was no significant association between MPOD and plasma CRP levels (Table 7.3).

Table 7.3: MPOD according to plasma biomarkers in a diabetic population (normalised MPOD).

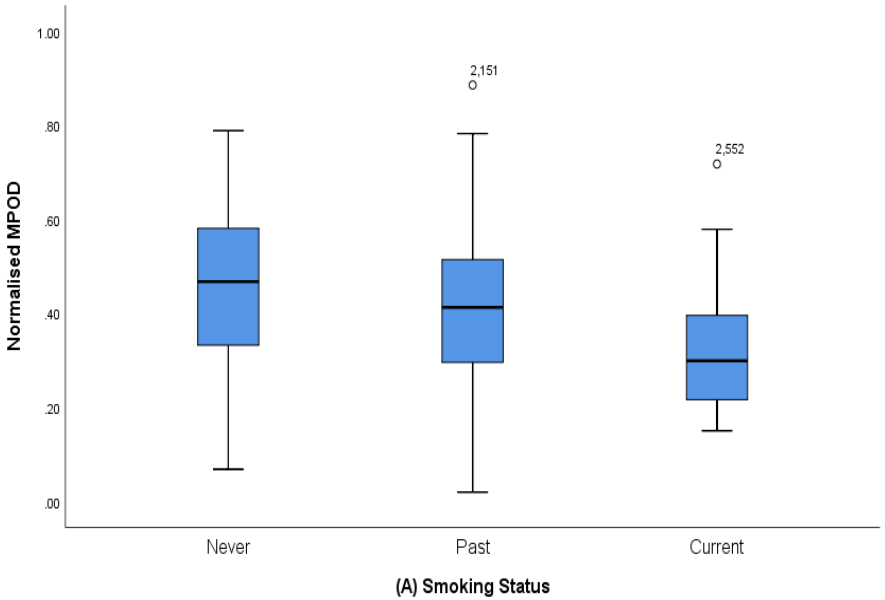
Variable	n (188)	Mean (SD) MPOD	25th	50th	75th	sig *p*
Plasma Biomarkers						
TC (mmol/L)						
Ideal (≤ 5.00)	141	0.207 (0.152)	0.088	0.182	0.304	0.287††
Ideal (> 5.00)	46	0.177 (0.138)	0.068	0.163	0.260	
HDL (mmol/L)						
Ideal (> 1.6)	27	0.203 (0.138)	0.089	0.184	0.275	0.785††
Low (≤ 1.6)	160	0.199 (0.151)	0.085	0.173	0.296	
LDL (mmol/L)						
Ideal (≤ 2.6)	127	0.20 (0.147)	0.076	0.179	0.297	0.946††
High (>2.6)	10	0.195 (0.151)	0.087	0.174	0.276	
TG (mmol/L)						
Ideal (≤1.7)	97	0.203 (0.147)	0.099	0.177	0.285	0.491††
High (>1.7)	90	0.195 (0.151)	0.069	0.179	0.301	
Non-HDL Cholesterol Ratio						
Ideal (≤ 3.4)	132	0.202 (0.146)	0.088	0.178	0.303	0.671††
High (>3.4)	55	0.194 (0.157)	0.072	0.172	0.263	

TG/HDL Cholesterol Ratio						
Ideal (≤1.74)	118	0.215 (0.152)	0.100	0.181	0.314	**0.039**††
High (>1.74)	69	0.172 (0.140)	0.053	0.119	0.248	
Vitamin D (nmol/L)						
Deficient (≤ 50)	96	0.173 (0.148)	0.056	0.181	0.234	**0.006**††
Sufficient (> 50)	91	0.226 (0.145)	0.103	0.209	0.336	
CRP (µg/l)						
Ideal (≤ 3.0)	113	0.213 (0.158)	0.088	0.181	0.298	0.120††
High (> 3.0)	74	0.178 (0.130)	0.069	0.167	0.262	
HbA1c (%)						
(≤ 6.00)	77	0.203 (0.160)	0.081	0.173	0.295	0.728††
(> 6.00)	97	0.190 (0.139)	0.081	0.177	0.259	

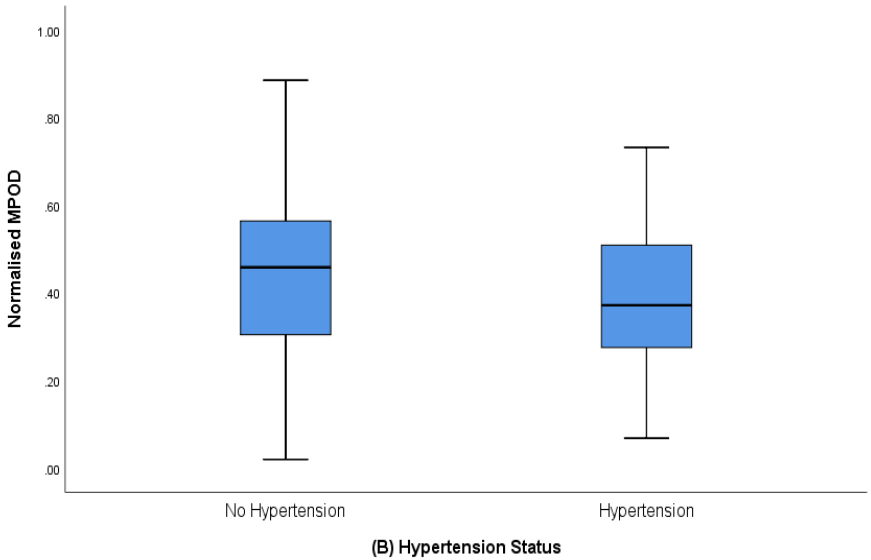
††ANOVA was used to check for MPOD differences among plasma biomarkers. P values are reported using MPOD square root transformation (sqrt) (normalised MPOD) and reflect the probability associated with the given F statistic. Sig., significance.

The following cut-offs were applied to plasma levels: HDL, high-density lipoprotein; [high risk ≤1.6; low risk >1.6 millimoles per litre (mmol/L)]; LDL, low-density lipoprotein; [high risk > 2.6; low risk ≤ 2.6 mmol/L]; TC, total cholesterol; [high risk > 5.00; low risk ≤ 5.00 mmol/L]; TG, triglycerides; [high risk >1.7; low risk ≤1.7 mmol/L];TG/HDL, triglyceride to high density lipoprotein ratio; [high risk >1.74; low risk ≤1.74 mmol/L]; TC/HDL, total cholesterol to high-density lipoprotein ratio; [high risk >3.5; low risk ≤3.5 mmol/L]; Non-HDL, total cholesterol minus high-density lipoprotein; [high risk >3.4; low risk ≤ 3.4 mmol/L] as per 2016 ESC/EAS guidelines;[528] plasma vitamin D (25(OH) D) levels in nanomoles per litre (nmol/L) [deficient ≤50; sufficient >50 nmol/L] as per IOM vitamin D guidelines;[530] plasma CRP levels in micrograms per litre (µg/l)

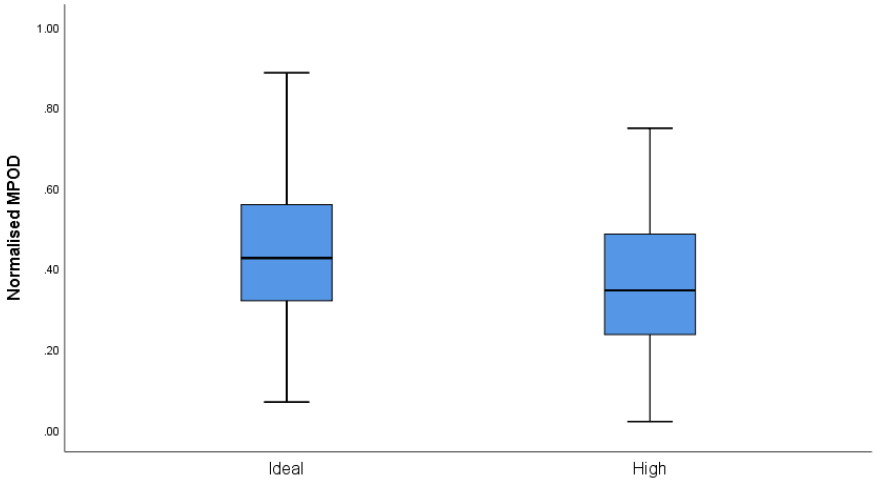
[high > 3.00 μg/L; ideal ≤3.00 μg/L];[531] and HbA1c (glycated haemoglobin) in millimoles per mol (mmol/mol) [high > 6.00 %; ideal ≤ 6.00 %] as per American Diabetes Association guidelines.[527]



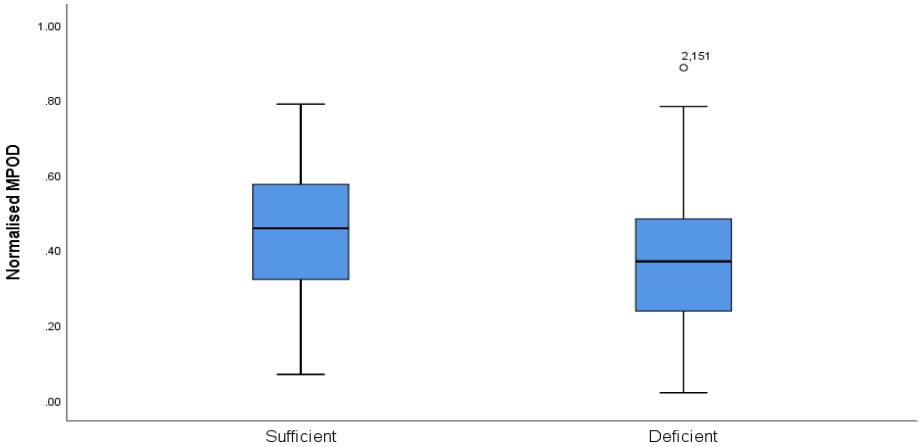
(A) Smoking Status



(B) Hypertension Status



(C) Triglycerides over High Density Lipoprotein Ratio



(D) Vitamin D (25(OH)D) Plasma Levels

Figure 7.4: Box plots of MPOD differences by (A) Smoking status (B) Hypertension status (C) Triglyceride over High Density Lipoprotein ratio (D) Vitamin D 25(OH) D plasma levels (ANOVA).

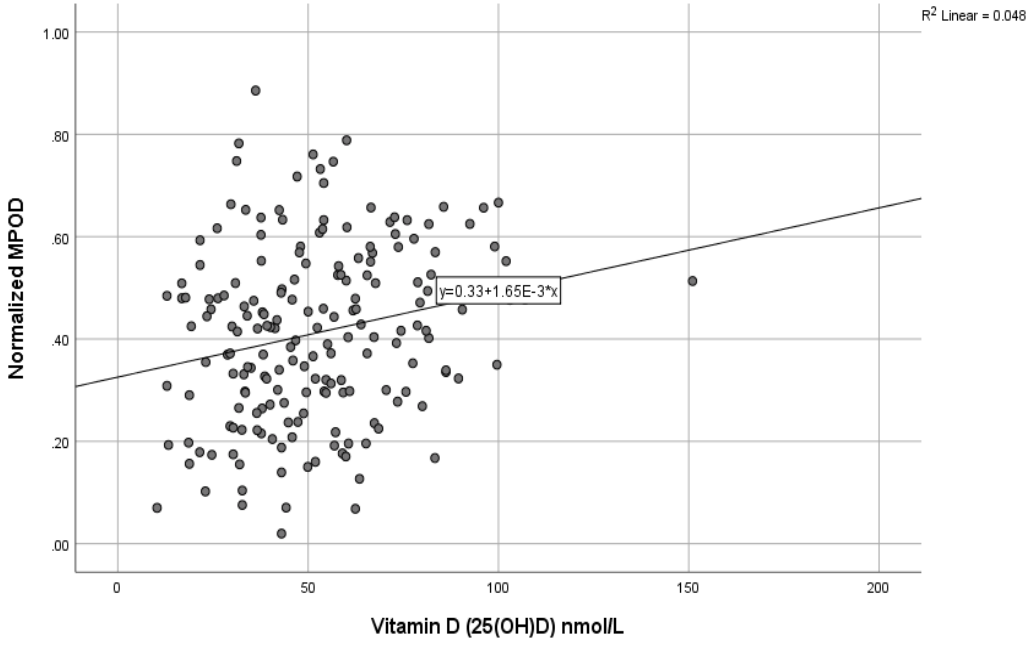


Figure 7.5: Scatterplot showing a significant positive relationship between normalised MPOD and Vitamin D (25(OH) D) plasma levels.

Multivariate model

Demographic and behavioural factors

Smoking remained a strong, negative predictor of MPOD after adjusting for covariates. Current smoking was significantly associated with lower MPOD (Beta coefficient = - 0.097; p=0.022; Table 7.4).

Anthropometric and physical biomarkers

The presence of cataract remained the only clinical parameter to be negatively associated with MPOD in a diabetic population after adjusting for covariates (Beta coefficient = - 0.078; p<0.049). None of the other anthropometric or clinical parameters (WHtR, hypertension, diabetic retinopathy or duration of disease) remained significantly associated with MPOD in this model (p>0.05 for all).

Plasma biomarkers

Vitamin D remained a significant and positive predictor of MPOD in the multivariate model, although this was a subtle effect (Beta coefficient = 0.001; p=0.029). None of the other plasma biomarkers (TG/HDL, HbA1c or CRP) remained significantly associated with MPOD (p>0.05 for these variables).

The correlates of MPOD identified as significant in the multivariate regression model (smoking, cataracts, and low vitamin D status) contributed 18.5% of the overall variability in MPOD status amongst patients with Type 2 diabetes, ($F(13,146) = 2.545$, p=0.003; $R^2 = 0.185$).

Table 7.4: Multivariate relationship between behavioural, anthropometric, clinical, plasma biomarkers and MPOD in a diabetic population (n=188); (F (13,146) = 2.545, p=0.003; R^2 = 0.185).

Independent Variable	Unstandardised Beta Coefficient	Std error	T	*p*
Constant	0.428	0.197	2.175	**0.031**
Age	0.001	0.002	0.700	0.485
Sex* (Female)	-0.007	0.025	-0.259	0.796
Smoking†				
Past	-0.028	0.031	-0.913	0.363
Current	-0.097	0.042	-2.316	**0.022**
WHtR	-0.192	0.183	-1.046	0.297
Cataracts‡ (Yes)	-0.078	0.039	-1.988	**0.049**
Vitamin D (nmol/L)	0.001	0.001	2.204	**0.029**
CRP (µg/l)	0.001	0.001	1.382	0.169
TG/HDL Cholesterol Ratio	-0.015	0.013	-1.170	0.244
HbA1c (%)	-0.006	0.017	-0.373	0.710
Duration (years)	0.003	0.003	1.093	0.276
Retinopathy†† (Yes)	-0.092	0.058	1.585	0.115
Hypertension ‡‡ (No)	0.035	0.027	1.302	0.195

R^2 = 0.185; F = 2.545; p= 0.003. Dependent variable = MPOD sqrt (normalised MPOD); Std. Error, Standard Error.

**Male = control group; † Never smoked = control group; ‡ No Cataracts = control group; ††No Diabetic Retinopathy = Control group; ‡‡ Hypertension= Control group.*

Abbreviations: CRP - C-reactive protein; HbA1c- glycated haemoglobin; µg/l – micrograms per litre; nmol/l – nanomoles per litre; WHtR- waist to height ratio; TG/HDL- triglyceride to high-density lipoprotein ratio.

7.5 Discussion

Consistent with previous investigations [6, 7] this study provides further evidence that patients with Type 2 diabetes have significantly lower MPOD, compared with non-diabetic controls. Additionally, MPOD was inversely associated with a range of behavioural, clinical and anthropometric biomarkers including smoking, hypertension, and bodyweight. Body mass index, WHtR, and WC were all negatively associated with MPOD on univariate analysis, in the Type 2 diabetes group, (Pearson's r, $p<0.05$ for all). Some novel relationships were observed among the plasma biomarkers, with low plasma vitamin D and raised TG/HDL ratio associated with low MPOD levels; findings not previously reported. Current smoking, the presence of cataract and low plasma vitamin D persisted as predictors of low MPOD in the multivariate model, after adjusting for all other covariates, albeit collectively explaining just 18.5% of the variability in MPOD.

Our finding that hypertensive participants with diabetes had significantly lower MPOD compared to normotensive participants with diabetes is important given that hypertension is an established risk factor for the development and progression of diabetic retinopathy.[533] The association between MP and hypertension is not well documented; however, one study has reported a link between lower MPOD and self-reported diagnosis of high blood pressure.[52] Chronic hypertension causes vascular endothelial shear stress and circumferential wall stress.[151] This, in turn, leads to endothelial damage, increased ROS production and activation of inflammatory cascades.[534] One recent study found lower levels of GSH and increased levels of 8-OHdG, a marker of oxidative stress, in diabetes patients with hypertension, supporting the hypothesis that oxidative stress increases considerably in hypertension, especially

as a diabetic co-morbidity.[535] Endogenous antioxidants become depleted when both conditions coexist, which leads to an increased need for exogenous antioxidants (*i.e.* vitamins C and E, carotenoids lutein, zeaxanthin, and *meso*-zeaxanthin) to balance redox status, which may explain our observation that hypertension in the presence of Type 2 diabetes may have an added negative impact on MPOD status on univariate analysis. While we did not reach statistical significance in multivariate analyses, future studies with larger cohorts could further investigate this finding.

The inverse relationship observed between adiposity and MPOD confirms previous findings among patients with Type 2 diabetes.[7] Adipose tissue, visceral fat in particular; may act as a sink/reservoir for macular carotenoids,[54] thereby influencing carotenoid concentrations in plasma,[347] and subsequent retinal uptake.[18] Distribution of body fat is also a significant factor, as it has been shown that concentrations of lutein and zeaxanthin are higher in abdominal fat,[67] and abdominal adiposity is characteristic in Type 2 diabetes. Excess visceral fat also contributes to inflammation and oxidative stress due to the increased production of adipocytokines (IL-1, IL-6, IL-8, and TNF-alpha).[372] The chronic low-grade inflammation, associated with obesity and Type 2 diabetes leads to increased oxidative stress and further inflammation, putting a greater demand on antioxidant defences. Antioxidant defences may also be lower in overweight/Type 2 diabetes patients, due to their lower intake of antioxidant-rich foods (e.g. fruits and vegetables), their increased utilisation of these molecules (e.g. increased inflammation/ROS in insulin-dependent tissue such as the retina) and their impaired generation of other supportive anti-oxidants, reviewed by Savini et al,[367] which may collectively lead to MPOD depletion.[68] Dietary intake of lutein and zeaxanthin was not, however, assessed as part of the TILDA study (this limitation is

discussed further below).

Of interest, and to our knowledge a novel finding; is that MPOD was significantly lower for participants with a raised TG/HDL ratio. This observation is important given that Type 2 diabetes patients commonly display an altered lipoprotein profile, termed diabetic dyslipidaemia (*i.e.* raised TGs and low HDL), and that diabetes patients in the current study had indeed significantly higher TGs and significantly lower HDL plasma lipid levels than the non-diabetic control group. The characteristic lipid profile of individuals with Type 2 diabetes,[158] may have important implications for MP levels in the eye, as dietary carotenoids are transported on circulating lipoproteins.[18] The general consensus is that xanthophylls associate with HDL,[327] and that HDL levels are therefore important for the efficient delivery and uptake of lutein and zeaxanthin to the eye.[18] The relationship between MPOD and plasma lipid levels has previously been investigated in patients with diabetes, with a marginal negative association between MPOD and TGs and a marginal positive association between MPOD and HDL observed ($p<0.08$ for both).[6] We previously reported significantly lower levels of MPOD in patients with Type 2 versus Type 1 diabetes, and noted significantly lower HDL values within the Type 2 diabetes group, postulating that lower HDL levels may have mediated this difference.[7] More recently, the DiVFuSS study,[21] has demonstrated significant improvements in plasma LDL, HDL, and TGs, in a group of patients with diabetes who participated in a 6-month RCT. The coincident increase in MPOD observed in the intervention group may have been partially mediated by these favourable changes in lipid profile.[18] We acknowledge, however, that the lack of dietary information on carotenoid intake is a limitation of the current study, (discussed in further detail below), as MPOD is influenced by both lutein and zeaxanthin intake

as well as transport and assimilation (*i.e.* mediated by HDL) in the target tissue. This information would have added to the interpretation of our findings. While we found that lower MPOD was associated with raised plasma TG/HDL levels on univariate analysis (p=0.039), plasma TG/HDL did not remain as a predictor of MPOD in multivariate analyses. Future studies, however, based on a larger number of diabetic participants could further investigate this finding.

Our findings in relation to smoking are not surprising given that smoking is associated with increased oxidative stress, not only through the increased systemic production of ROS but also through a weakening of the antioxidant defence systems.[65] The reported associations between cigarette smoking and diabetic retinopathy, however, are more variable, with some studies reporting an association,[536] while others have found no such relationship.[537] Chronic hyperglycaemia causes oxidative stress, so the increased production of ROS in diabetes may be compounded further by smoking, another important source of free radicals. Exposure to cigarette smoking causes profound oxidative damage to human RPE cells.[538] Interestingly, we found no difference in MPOD between previous smokers and non-smokers, perhaps indicating that MPOD repletion is possible in diabetes patients through smoking cessation.

Cataract status remained a predictor of low MPOD in the multivariate model in line with previous observations.[260, 539] The marginal statistical significance of this association in the univariate model (p=0.068) was more than likely limited by the small sample size in the current study (n=26 with cataracts). The evidence that carotenoids, lutein, and zeaxanthin, are protective against the development of cataract, however, is well documented [260, 539] suggesting the possibility of a causal role for

MPOD depletion in the pathogenesis of cataract development in diabetes. With ageing, there is a natural decrease in the production of antioxidants and antioxidant enzymes and a concomitant increase in phototoxic chromophores in the lens.[250] Phototoxic reactions, whether caused by endogenous or exogenous singlet oxygen photo-sensitisers, lead to a modification of lens proteins, which eventually causes opacification of the lens (*i.e.* cataractogenesis) (reviewed by Roberts & Dennison [250]). The phototoxic reaction damage can be prevented by the appropriate antioxidant quenchers. Lutein and zeaxanthin both accumulate in the lens [540] and research has shown a lower prevalence of nuclear cataract amongst those with higher intakes of xanthophylls.[539] Furthermore, findings from the AREDS2 revealed that participants in the lowest quintile of dietary lutein/zeaxanthin intake experienced slower cataract progression with carotenoid supplementation.[260] Although the development of cataract is an age-related phenomenon, other factors appear to influence their progression including poor diet, smoking, UV light exposure, and metabolic factors. In the current study, a greater percentage of patients with diabetes had cataract (13.83%) compared with controls (6.51%), (p<0.0001). Cataract status does not appear to relate to MPOD levels among non-diabetic individuals,[52] suggesting a relationship that is particular to cataract in the presence of Type 2 diabetes, which may impact MPOD levels in this patient group. To our knowledge, this is the first study to report an association between low MPOD and the presence of cataract in diabetes.

That vitamin D emerged as a positive predictor of MPOD in Type 2 diabetes after controlling for other covariates represents a particularly novel and interesting finding. Although the best-known function of vitamin D is to help the body absorb and use calcium, other important roles include protection of the eye during inflammation,

oxidative stress, fibrosis, and angiogenesis.[541] Vitamin D insufficiency is common amongst the general population, and particularly amongst those who are obese, [542] or who have diabetes.[543] Our findings support this observation as both the control and Type 2 diabetes group had mean vitamin D levels which fell well below the optimal recommended level,[544] and as the participants with Type 2 diabetes had significantly lower plasma vitamin D compared with the control group ($p<0.0001$). Vitamin D receptors are expressed in the eye, including the RPE,[545] which suggests that vitamin D is biologically relevant to the eye.[307] Research shows that human adult RPE cells (ARPE-19) also express the 1α-hydroxylase enzyme required to convert 25(OH) D to its biologically active 1,25 $(OH)_2$ D form.[546]

The ability to inhibit neovascularisation has also led researchers to examine vitamin D's involvement in diabetic retinopathy development.[547] Plasma vitamin D concentrations have previously been found to be inversely related to the severity of retinopathy in patients with diabetes,[307] with this study suggesting that the measurement of serum 1,25$(OH)_2 D_3$ concentrations might be helpful in predicting retinopathy progression, and that a detailed ophthalmologic examination is indicated for diabetes patients whose plasma vitamin D levels are low.[307] The DiVFuSS study recently examined the effects of supplementation with a novel multi-component nutritional supplement, which included vitamin D and the antioxidants lutein and zeaxanthin, on ocular health and visual function in a group of participants with diabetes.[21] Individuals receiving the DiVFuSS formula improved on all measures of visual function, and although not statistically significant ($p=0.07$), four subjects were downgraded from moderate to mild non-proliferative diabetic retinopathy following the intervention, while one subject on placebo was upgraded from mild to moderate

non-proliferative diabetic retinopathy over the same period.[21] Positive outcomes from DiVFuSS may in part be due to supplementation with a compound that specifically targets both inflammation and oxidative stress. It is plausible that these findings may have been mediated by enhancements in MPOD (27% mean increase in DiVFuSS group vs 2% mean decrease in placebo group), and by vitamin D's attenuating effect on inflammation with reductions in plasma hs-CRP (60% mean decrease in DiVFuSS group vs 11% mean decrease in placebo group).[21] Although CRP levels were significantly higher in participants with diabetes [mean =5.23(11.94)], compared with non-diabetic controls [mean=2.83(6.95)], (p=0.0001), in our study, MPOD was not significantly different in diabetic participants with an elevated CRP level compared with ideal levels (p=0.120; Table 7.3). These findings, however, warrant further investigation.

7.6 Limitations

There are a number of important limitations to the current study which should be recognised. Firstly, the use of self-reported data is not ideal. Diabetes diagnosis was based on respondent's recall rather than health records. Concomitantly, respondents were not explicitly asked what type of diabetes they had. Instead, patient medications (self-reported) and age at diagnosis were used to account for possible Type 1 cases, which may have led to some misclassification. The presence or absence of cataract was also based on patient's recall rather than direct examination. Secondly, small patient numbers in some of the sub-group analyses may have limited the statistical power to detect associations that may exist (Type II error possibility). It is worth noting that the prevalence of diagnosed and undiagnosed Type 2 diabetes in these older adults resident in Ireland was found to be relatively low compared to other populations (8.6% and 0.9% respectively), which would account for low participant numbers in the

current study.[130, 520] Another limitation was that dietary intake of the macular carotenoids was also not assessed, therefore, it was not possible to control for potential confounding from variable intake of lutein and zeaxanthin. Macular pigment constituent carotenoids cannot be synthesised *de novo* in humans, therefore, lower levels of MPOD may be experienced in participants with poor dietary intake of antioxidants. Mares et al,[384] found that MPOD was directly related to dietary intake of carotenoids, lutein, and zeaxanthin, but even more strongly with plasma levels, suggesting that unmeasured physical and medical factors may also influence the uptake, distribution and utilisation of lutein and zeaxanthin.[384] The absence of plasma analysis of lutein and zeaxanthin was also a weakness in our study.

Furthermore, it is worth noting that measures of MP are subject to genetic variation. Macular pigment optical density is a multi-factorial phenotype, associated with variation in genes related to carotenoid transport, uptake, and metabolism, and may be independent of known dietary and health influences in MPOD.[72] The MP determinants reported herein (current smoking, presence of cataracts and vitamin D status) explained 18.5% of the overall variability in MPOD, in participants with diabetes. Diet is an important determinant of MPOD, therefore, cautionary interpretation of our findings is advised. Finally, while the current study used a large, nationally representative population, MPOD was only measured on participants who were able to attend a health centre.

Despite these drawbacks, there were a number of strengths to the current analysis. The design of the TILDA study is a particular strength, in particular, the selection of participants from which our diabetes and controls were drawn, which was

representative of the Irish population aged 50 and over. Plasma lipoproteins (HDL, TG/HDL) and inflammatory markers (vitamin D, CRP) were analysed and blood pressure was measured, which represents an advance on previous research carried out, research which was based on self-reported doctors' diagnosis of high cholesterol and hypertension.[52] The additional use of HbA1c helped identify undiagnosed and pre-diabetes cases. Finally, anthropometric measures such as WHtR and WHpR were used to analyse overweight/obesity in conjunction with BMI and WC.

7.7 Conclusion

Overall our findings suggest that individuals with Type 2 diabetes have lower MP relative to healthy controls, although the clinical importance of the observed level of difference is questionable. Future studies with larger cohorts could further investigate this finding, as the difference in MPOD, although significant (p=0.047), was marginal. Hyperglycaemia and other anthropometric, metabolic and clinical correlates associated with diabetes (e.g. excess adiposity, dyslipidaemia, hypertension, and cataract), may relate to a state of chronic oxidative stress and inflammation, processes which also underlie many of the functional alterations in the retinal vasculature in diabetic retinopathy. Both over utilisation (*i.e.* in response to elevated oxidative stress/inflammation) and undersupply of antioxidant nutrients (dietary deficiency, excess adiposity, and dyslipidaemia) may contribute to lower levels of MP in the diabetic retina. Clinical benefits may be realised through the early identification of MPOD depletion in Type 2 diabetes. Given the borderline statistical significance of many of our findings, more work needs to be done to verify and refine our understanding of the observed relationships. The MPOD difference of 0.053 OD in participants with sufficient vitamin D (>50nmol/L) versus deficient levels (≤50 nmol/L), although significant (p<0.006), is not clinically meaningful as it stands.

Macular pigment optical density measurement is not routinely available, however, the capacity of commonly-measured surrogate plasma biomarkers (HDL, TG/HDL, vitamin D), and anthropometric measurements (WHtR, WC) to identify people with diabetes at risk of low MP merits further consideration. The novel and important findings reported herein should now be subject to further research, to better understand the nature of any relationships that may exist.

8. INVESTIGATION OF SURROGATE BIOMARKERS ASSOCIATED WITH LOWER MACULAR PIGMENT IN A GROUP OF OLDER IRISH ADULTS.

8.1 Abstract

Purpose

Macular pigment confers potent antioxidant and anti-inflammatory effects at the macula but its optical density in the eye is not routinely measured in clinical practice. This study explored a range of anthropometric, clinical, and plasma measures which are commonly measured and which may be associated with lower MPOD, in a large, population-based sample of older adults.

Method

Two thousand five hundred and ninety four subjects completed a full MP assessment, as part of Wave 1 of TILDA. The MPOD was measured using c-HFP. Clinical [blood pressure], plasma [lipoproteins, inflammatory markers] and anthropometric [waist (cm), hip, (cm), height (cm) weight (kg)] biomarkers were measured for each participant.

Results

Mean (SD) MPOD for the study group was 0.223 (0.161) with a range of 0 to 1.08. One way analysis of variance revealed that MPOD was significantly lower among participants with: low plasma HDL (p =0.038), raised plasma TG/HDL ratio (p=0.003) and raised TC/HDL ratio (p=0.030). Subjects with an elevated WC had significantly lower MPOD [mean = 0.216(0.159)] compared to those with an ideal WC [mean = 0.229(0.162); p=0.034]. Significant correlates of MPOD on mixed linear model

analysis included education, smoking status and WC.

Conclusions

Higher abdominal fat is associated with lower MPOD in this representative sample of older Irish adults. While altered lipoprotein profiles (low HDL, raised TG/HDL ratio, raised TC/HDL ratio), may affect the transport, uptake and stabilisation of carotenoids in the retina, these plasma biomarkers were not predictive of low MPOD after adjustment for abdominal circumference. Although WC emerged as a possible anthropometric predictor of lower MPOD, its effect size appears to be small and clinical applicability questionable.

8.2 Introduction

The concept that MP may have a protective role in neurodegenerative eye conditions such as AMD, glaucoma and diabetes is well documented.[4, 353, 548] The macula is particularly important for central high resolution vision and blindness results when this area is lost to disease. The carotenoids lutein, zeaxanthin, and *meso*-zeaxanthin, collectively known as MP, accumulate at the macula to the exclusion of all other dietary carotenoids. Macular pigment has a unique distribution within the retina, selectively located within the fibres of Henle in the fovea and in the inner nuclear layer of the parafovea,[43, 549] implying that MP plays an important role in vision and macular health, and that it is biologically relevant to the eye.[44, 45]

The selective absorption of short-wavelength light prior to photoreceptor light capture means that MP plays an important role in visual performance in healthy eyes.[2] Concomitantly, through its optical filtration and antioxidant properties, MP protects the retina from photo-oxidative damage, thereby, reducing the risk of various eye diseases, including AMD [5] and diabetes.[15, 21] Decreased MPOD appears to be a risk factor for the development of these diseases,[6, 7, 55] and numerous studies investigating the effects of MP augmentation have also reported beneficial effects in diseased eyes.[5, 15, 218] While the aetiopathogenesis of conditions such as AMD and diabetes remain a matter of debate, there is growing consensus that oxidative damage,[291, 355] and associated inflammation [10, 242] play a significant role. The macular carotenoids exhibit neuroprotective functions at the macula,[4, 230] and it has been shown that lutein and zeaxanthin can affect immune responses, reduce inflammation and have anti-angiogenic properties in the eye.[4, 550, 551] Therefore, the density of MP, offers potential as a clinical biomarker of retinal health.

Macular pigment levels can be measured *in vivo*, but the relevant instrumentation is not commonly available in clinical practice. Other indirect techniques for assessing MP status such as dietary and plasma analysis have limited practical application and are not used in clinical practice. Previous investigators have examined and identified possible predictors of MPOD, and found associations with dietary intake,[384] plasma cholesterol and lipoprotein status,[18, 71] metabolic status,[6, 7] overweight/obesity status,[52] and smoking.[52] Many of these studies, however, were based on opportunistic sampling and restricted to small sample sizes,[6, 7, 71] or relied on self-reported data.[52] While one study investigated determinants of MPOD on a larger older cohort (n=1698), this research was conducted on female subjects only.[384]

Consequently, there is merit in exploring the association between MPOD and more commonly measured clinical and biometric parameters, which might be used to identify patients at risk of low MP, so that practitioners can implement strategies for preventative intervention. While self-reported data provide a reasonable measure of overall health status, concerns exist regarding the magnitude of bias from using these measures in analysis.[552, 553] This study was designed, therefore, to address some of the limitations of previous investigations by examining the predictive capacity of a range of measured (rather than self-reported) clinical (blood pressure), plasma (lipoproteins, inflammatory markers) and anthropometric (abdominal fat) parameters in a large representative group of older men and women.[523]

8.3 Methods

Study design and population

Cross-sectional data from Wave 1 of TILDA were analysed in this study, which was

conducted during the period between October 2009 and July 2011. A sample of 8,175 individuals representative of the population of Ireland aged 50 years and older participated in the study, as described in detail elsewhere.[523, 524] In brief, information on health behaviours and lifestyle patterns were captured by trained professional interviewers in participants' own homes using CAPI.[525] Participants were then invited to take part in a health assessment.[526] Macular pigment measurement was only conducted on participants who attended a health centre (Figure 8.1). In total 5,275 consented to and participated in the health centre based assessment. The current study was approved by the Technological University Dublin Research Ethics Committee and all experimental procedures adhered to the tenets of the Declaration of Helsinki. All participants provided written informed consent prior to participation in the study.

Demographic, health and lifestyle factors

Demographic, health, and lifestyle factors were captured for the study group. The presence or absence of eye pathology was determined using the CAPI with the question ''has a doctor ever told you if you had any of the following conditions: diabetes, AMD, cataracts or glaucoma?''. Participants were asked if they currently smoked or had ever smoked cigarettes regularly (daily for at least a year), and were categorised as never smoked, past smokers or current smokers. Blood pressure was measured and participants were defined as hypertensive if mean seated systolic BP exceeded 140 mmHg or mean seated diastolic BP exceeded 90 mmHg.

Macular pigment optical density assessment

Macular pigment optical density was measured by c-HFP using the Macular Metrics Densitometer (Macular Metrics, Rehoboth, MA). This device was modified specifically for the TILDA study and the method of measurement has been described

in detail elsewhere.[56] In brief, MPOD was measured centrally at 0.5° (*i.e.* 1° stimulus) and peripherally at 7° (parafoveal target at 7°). Macular pigment assessment was carried out on the eye with the best VA or if there was equal vision in both eyes the right eye was chosen.[56] Corrected VA was measured using the ETDRS LogMAR chart at a distance of 4 metres using participants' existing prescription where necessary. Participants with a VA worse than 0.5 LogMAR were excluded from the analysis (Figure 8.1).

Anthropometric assessment

Height (cm) and weight (kg) were measured to one decimal place using a Seca wall mounted measuring rod and Seca electronic floor scales, as described in detail elsewhere.[525] Body mass index was calculated from measured height and weight as: weight (kg)/height (m^2), with obesity classified as a BMI > 30 kg/m^2.[363] Waist and hip circumferences were measured to the nearest cm. Ideal WC was defined as a WC of ≤ 88 cm in women and ≤ 102 cm in men, while central obesity was defined as a WC > 88 cm in women and > 102 cm in men. Waist-to-height ratio and WHpR were calculated based on measured data. Cut-off thresholds for ideal and excess WHtR were taken as less than or greater than 0.53 in women and 0.57 in men respectively. For WHpR a cut off threshold of less than or greater than 0.85 in women and less than or greater than 1.00 in men defined ideal and elevated WHpR.[527, 554]

Plasma analysis

A blood sample was provided for plasma analysis using defined phlebotomy protocols, which are described in detail elsewhere.[525, 526] Respondents were not asked to fast before the health assessment and plasma was analysed for a complete lipid profile, including TC, HDL, LDL and TGs, each measured in mmol/L. Triglyceride over HDL

ratio, TC/HDL ratio and non-HDL cholesterol were calculated from measured data. Cut-offs were applied to indicate high/low or ideal plasma lipid levels as per 2016 ESC/EAS guidelines (Table 8.2).[528] Plasma vitamin D levels [(25(OH)D); nmol/L] were also measured and participants were considered vitamin D deficient if plasma levels were ≤50 nmol/L and sufficient if plasma levels were >50 nmol/l as per IOM vitamin D guidelines.[530] Inflammatory marker CRP [μg/l] and HbA1c (%) were also measured. A threshold of > 3.00μg/l for high and ≤ 3.00μg/l for ideal was used for plasma CRP [531] and a cut off of ≤ 5.00% and > 5.00% was used for HbA1c.

Statistical analysis

The statistical software package SPSS for Microsoft Windows (v.25.0; IBM Corp., Armonk, NY) was used for analysis. To account for the fact that the study response rate varied between different subgroups of the population, inverse probability weights were calculated for the main sample (CAPI participants) using the Quarterly National Household Survey 2010. The probability of participation in the health centre also varied according to health, education, age and smoking, therefore, a specific 'health centre weight' was created. A detailed description of the weighting procedures used in TILDA is given elsewhere.[532]

Demographic data for eligible study participants were compared to those of the overall health centre population using Chi-square analyses, to explore if they differed from the representative sample. Data for the study group were tested for normality using the Kolmogorov-Smirnov test. One-way analysis of variance was used to test for differences in means for normally distributed parameters. For categorical data, cross-tabulation with Chi-square analysis was used. Pearson's product-moment correlation tests were performed to assess the relationship between normalised MPOD and other

study variables where appropriate. A mixed linear model analysis (fixed effect) was carried out to estimate the independent association between putative predictor variables (behavioural, clinical, anthropometric, and plasma biomarker parameters) and normalised MPOD. Data are presented as mean ± standard deviation throughout. The level of statistical significance was set at $p< 0.05$ for all analyses.

8.4 Results

In total, 8,175 individuals aged 50 years and older participated in the study. Of these, 5,275 consented to and participated in the health centre based assessment as part of Wave 1 of TILDA. For various reasons, 2,681 were excluded from the current analysis, leaving an overall study population of 2,594 (Figure 8.1). The main reasons for exclusion related to unsuccessful MPOD measurement or the presence of retinal pathology. Participants were identified with AMD, cataracts and glaucoma from the CAPI. The presence or absence of diabetes was identified from prescribed diabetes medications at the time of the interview (identified using the ATC codes 'A10A' for insulin and 'A10B' for oral hypoglycaemic medications), from the CAPI and/or from measured HbA1c [HbA1c > 6.4% (diabetes); 5.7 - 6.4% (pre-diabetes)], as per American Diabetes Association cut-off values.[24]

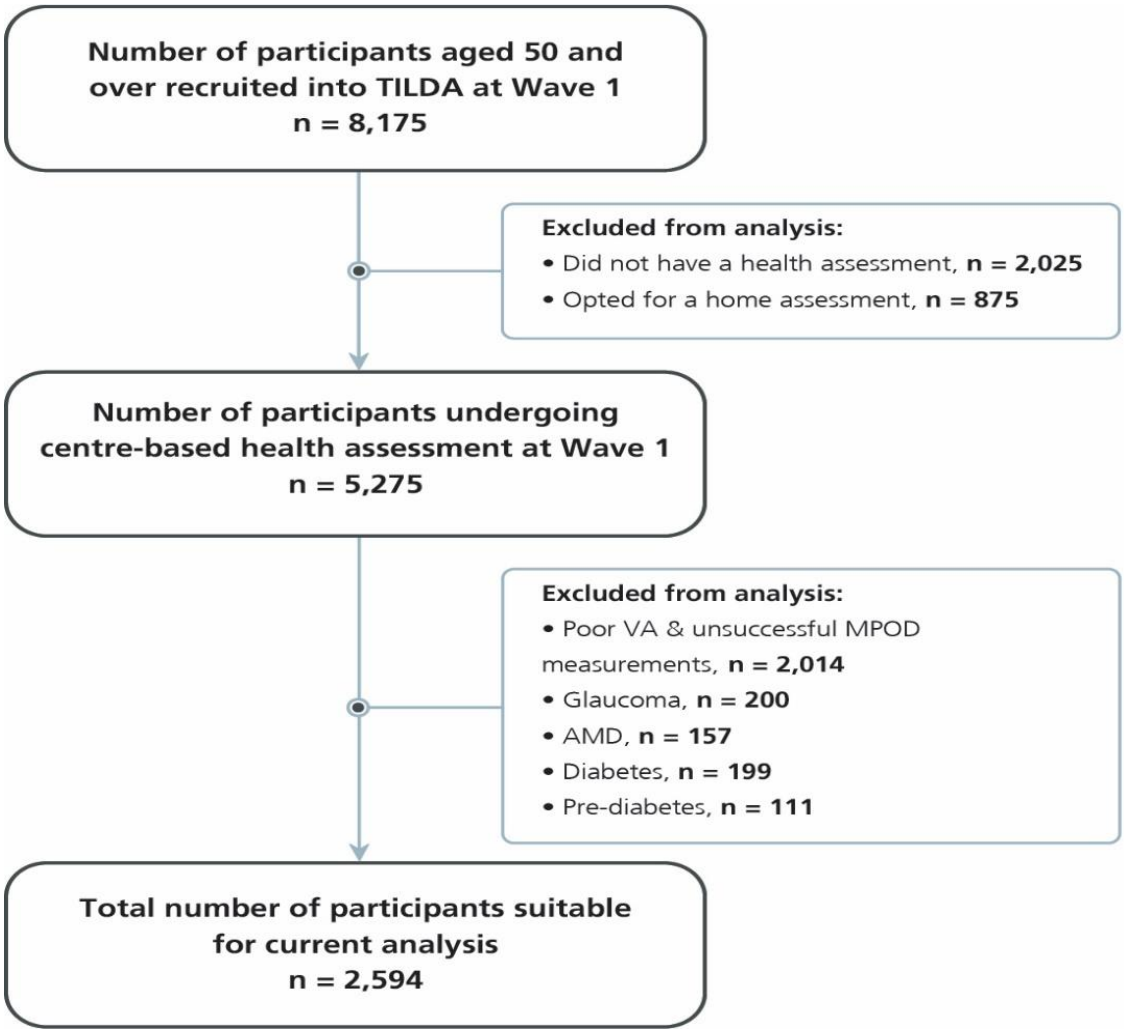


Figure 8.1: Flowchart illustrating the selection of study participants and reasons for exclusion from the original participant group.

Abbreviations: AMD, age related macular degeneration, MPOD, macular pigment optical density, VA, visual acuity, TILDA, the Irish Longitudinal Study on Ageing.

A Chi-square test of independence was performed to examine the relationship between the centre population (n=5,275) and participants with a valid MPOD assessment (n=2,594). The age profile of study participants [mean age = 61.95 (8.47) years; range 50 to 93 years] was similar to the overall health centre assessed population [mean age = 61.40 (7.62) years; range 51 to 86 years] and there was no significant difference in

sex or education status between the two groups (p=0.882 and p=0.768 respectively). Mean (SD) MPOD for the study group was 0.223 (0.161) with a range of 0 to 1.08. Differences in normalised MPOD by demographic (age group, sex, education status), health and lifestyle variables (smoking, hypertension, physical activity and cataracts) were examined. Participants were divided into three age groups: 50-64 years (mean MPOD =0.223[0.160]); 65 to 74 years (mean MPOD=0.223[0.164]); and 75 years and over (mean MPOD =0.216[0.151]). Age was not significantly associated with MPOD on one-way ANOVA (p=0.852). Additionally, age as a continuous variable was not significantly associated with MPOD (r=-0.003, p=0.883). Our findings concur with previously reported statistically significant associations between gender, educational status and smoking with MPOD in the TILDA dataset (p<0.05 for all), despite a slightly different study sample (participants with AMD, glaucoma, diabetes and pre-diabetes were excluded from the current analysis of older individuals, but not in a previous study).[52]

Clinical, anthropometric and biomarker associations with MPOD

(1) Clinical factors

We found no correlation between measured hypertension status (with or without treatment) and MPOD in the current study [n= 2,591; t=1.105; p=0.331].

(2) Anthropometric factors

The MPOD was significantly and negatively correlated with all anthropometric measures (BMI; WC; WHtR and WHpR) on univariate analysis (Pearson's r = -0.047; -0.073; -0.060; -0.081 respectively, p<0.05 for all). Waist circumference, however, emerged as the only anthropometric variable associated with lower MPOD on binary univariate analysis. Participants with an elevated WC had significantly lower MPOD

[Mean =0.216(0.159)] compared to participants with ideal WC [Mean = 0.229(0.162); n=2,589; t=2.119; *p*=0.034; Table 8.1; Figure 8.2], although the differences were not clinically meaningful, as illustrated in Figure 8.2. MPOD did not differ significantly according to any other anthropometric measures ($p>0.05$ for all; Table 8.1).

Table 8.1: The MPOD according to anthropometric biomarkers in the study population (n=2594); (normalised MPOD).

Variable	n (2594)	Mean (SD) MPOD	25th	50th	75th	Sig *p*
Anthropometric Biomarkers						
BMI (kg/m^2)						
Ideal (≤30)	1387	0.223[0.161]	0.100	0.191	0.310	0.829
Excess (>30)	1203	0.223[0.160]	0.101	0.195	0.316	
WC (cm)						
Ideal (≤102 M, ≤88 F)	1433	0.229[0.162]	0.105	0.196	0.319	**0.034***
Excess (>102 M, >88 F)	1158	0.216[0.159]	0.096	0.187	0.301	
WHpR						
Ideal (≤ 1.00 M, ≤0.85 F)	1637	0.225[0.158]	0.105	0.195	0.314	0.133
Excess(>1.00 M, >0.85 F)	954	0.219[0.165]	0.093	0.189	0.307	
WHtR						
Ideal (≤0.57 M, ≤0.53 F)	1307	0.228[0.161]	0.107	0.197	0.317	0.079
Excess (>0.57 M, >0.053 F)	1282	0.219[0.162]	0.095	0.188	0.306	

One way ANOVA was used to check for MPOD differences among anthropometric and physical biomarkers. P values are reported using MPOD square root transformation (sqrt) (normalised MPOD) and reflect the probability associated with the given F statistic. Sig., significance.

The following cut-offs were applied to indicate ideal or excess obesity measures and normotensive/hypertension for the following variables:

BMI-body mass index; [ideal ≤ 30; excess > 30 kg/m^2 - kilograms per metre squared; WC-waist circumference; ideal ≤ 88 F; ≤ 102 M;

excess > 88 F; >102 M cm - centimetres; WHtR-waist-to-height ratio; ideal ≤ 0.53 F; ≤ 0.57 M; excess >0.57 F; >0.53 M;

WHpR-waist-to-hip ratio; ideal ≤0.85 F; ≤1.00 M; excess > 0.85 F; >1.00 M; M=Male; F=Female.

Abbreviations: BMI- body mass index; cms - centimetres; F-female; kg-kilogram; M-male; MPOD-macular pigment optical density;

WC-waist circumference; WHpR – waist-to-hip ratio; WHtR – waist-to-height ratio.

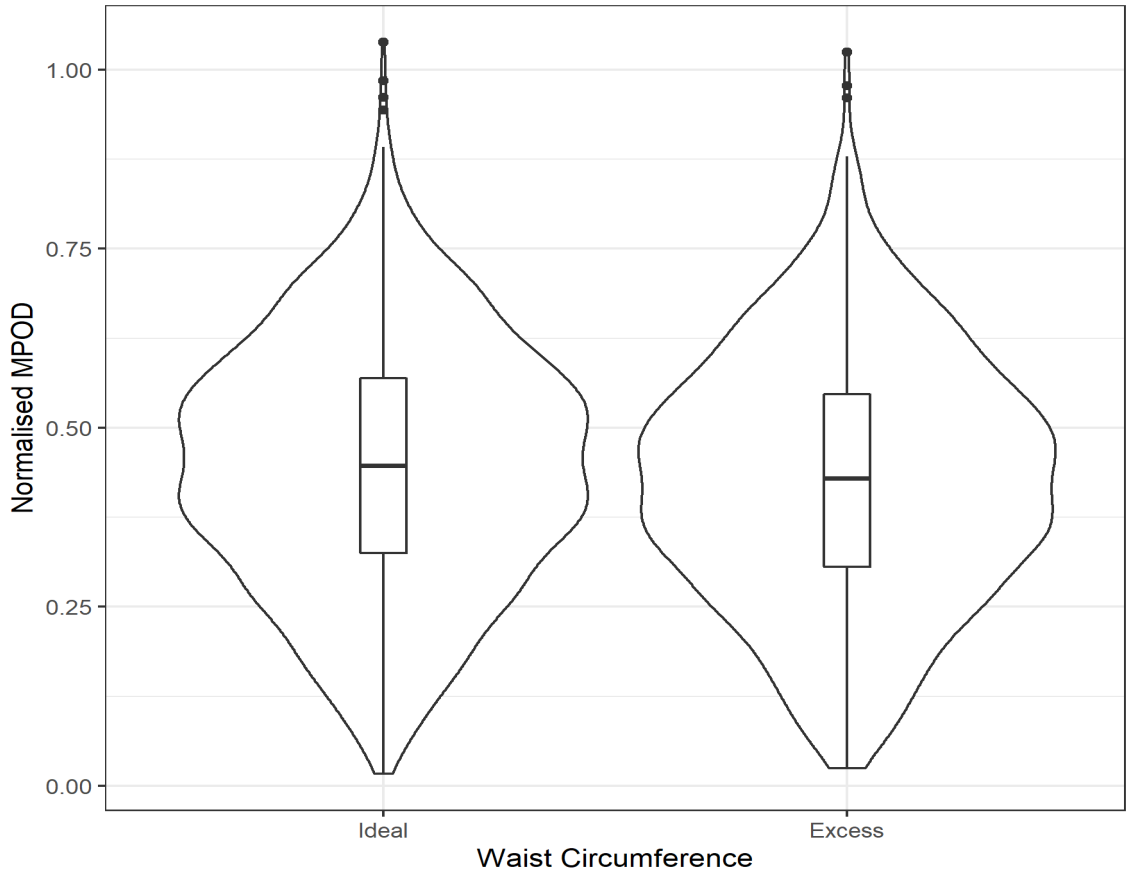


Figure 8.2: Violin plot of MPOD differences according to waist circumference status (ANOVA).

(3) Plasma biomarkers

Participants with ideal plasma levels of HDL had significantly higher MPOD than those with low HDL levels [n=2587; t=-2.077; *p*=0.038; Table 8.2; Figure 8.3 (A)]. The MPOD was also significantly lower among participants with a raised TG/HDL ratio [n=2587; t = 2.994, *p*=0.003; Table 8.2; Figure 8.3 (B)] and among participants with a raised TC/HDL ratio [n=2574; t=2.166; *p* =0.030; Table 8.2; Figure 8.3 (C)]. The magnitude of all statistically significant differences were not, however, clinically important, as illustrated in Figure 8.3. One-way analysis of variance revealed no significant difference in MPOD levels for any other plasma biomarkers (*p*>0.05 for all); Table 8.2**).**

Table 8.2: The MPOD according to plasma biomarkers in the study population (n=2594), (normalised MPOD).

Variable	n (2594)	Mean (SD) MPOD	25th	50th	75th	Sig *p*
Plasma Biomarkers						
TC (mmol/L)						
Ideal (≤5.00)	1040	0.224[0.161]	0.109	0.192	0.315	0.735
High (>5.00)	1549	0.222[0.160]	0.096	0.192	0.312	
HDL (mmol/L)						
Ideal (>1.6)	1126	0.229[0.160]	0.104	0.203	0.323	**0.038***
Low (≤1.6)	1463	0.218[0.161]	0.096	0.183	0.305	
LDL (mmol/L)						
Ideal (≤2.6)	833	0.222[0.157]	0.109	0.190	0.314	0.965
High (>2.6)	1756	0.223[0.162]	0.098	0.193	0.312	
TG (mmol/L)						
Ideal (≤1.7)	1625	0.224[0.161]	0.102	0.192	0.313	0.487
High (>1.7)	961	0.220[0.161]	0.097	0.191	0.312	
Non HDL (mmol/L)						
Ideal (≤3.4)	1044	0.224[0.159]	0.108	0.190	0.314	0.715
High (>3.4)	1541	0.222[0.162]	0.098	0.194	0.311	
TG/HDL Cholesterol Ratio						
Ideal (≤0.87)	1187	0.233[0.163]	0.106	0.204	0.322	**0.003***

High (>0.87)	1402	0.214[0.158]	0.095	0.182	0.304	
TC/HDL Cholesterol Ratio						
Ideal (≤3.5)	1483	0.229[0.164]	0.105	0.200	0.319	**0.030***
High (>3.5)	1093	0.214[0.156]	0.096	0.180	0.300	
Vitamin D (nmol/L)						
Low (≤ 50)	963	0.223[0.159]	0.107	0.191	0.305	0.654
Optimal (> 50)	1609	0.222[0.162]	0.096	0.192	0.314	
CRP (µg/l)						
Low (≤ 3.0)	1927	0.224[0.158]	0.103	0.196	0.314	0.186
High (>3.0)	644	0.218[0.170]	0.093	0.181	0.305	
HbA1c (%)						
≤ 5.00	1206	0.221[0.159]	0.101	0.188	0.309	0.633
>5.00	1388	0.225[0.162]	0.101	0.196	0.316	

One way ANOVA was used to check for MPOD differences among plasma biomarkers. P values are reported using MPOD square root transformation (sqrt) (normalised MPOD) and reflect the probability associated with the given F statistic. Sig., significance.

The following cut-offs were applied to plasma levels: HDL, high density lipoprotein; [high risk ≤1.6; low risk >1.6 millimoles per litre (mmol/L)]; LDL, low density lipoprotein; [high risk > 2.6; low risk ≤ 2.6 mmol/L]; TC, total cholesterol; [high risk > 5.00; low risk ≤ 5.00 mmol/L]; TG, triglycerides; [high risk >1.7; low risk ≤ 1.7 mmol/L];TG/HDL, triglyceride to high density lipoprotein

ratio; [high risk >0.87; low risk ≤ 0.87 mmol/L]; TC/HDL, total cholesterol to high density lipoprotein ratio; [high risk >3.5; low risk ≤ 3.5 mmol/L]; Non-HDL, total cholesterol minus high density lipoprotein; [high risk >3.4; low risk ≤ 3.4 mmol/L] as per 2016 ESC/EAS guidelines;[528] plasma vitamin D (25(OH) D) levels in nanomoles per litre (nmol/L) [deficient ≤ 50; sufficient >50 nmol/L] as per IOM vitamin D guidelines;[530] plasma CRP levels in micrograms per litre (µg/l) [high risk > 3.00 mg/L; low risk ≤ 3.00 µg/l];[531] and HbA1c (glycated haemoglobin) in millimoles per mol (mmol/mol) [high > 5.00 %; ideal ≤ 5.00 % (42.1 mmol/mol) as per American Diabetes Association guidelines.[24]

Abbreviations: CRP - C-reactive protein; HbA1c- glycated haemoglobin; HDL-high density lipoprotein; LDL-low density lipoprotein; µg/l – micrograms per litre; MPOD-macular pigment optical density; non HDL –TC-HDL; nmol/L – nanomoles per litre; TC-total cholesterol; TC/HDL- total cholesterol over high density lipoprotein; TG-triglyceride; TG/HDL- triglyceride over high density lipoprotein.

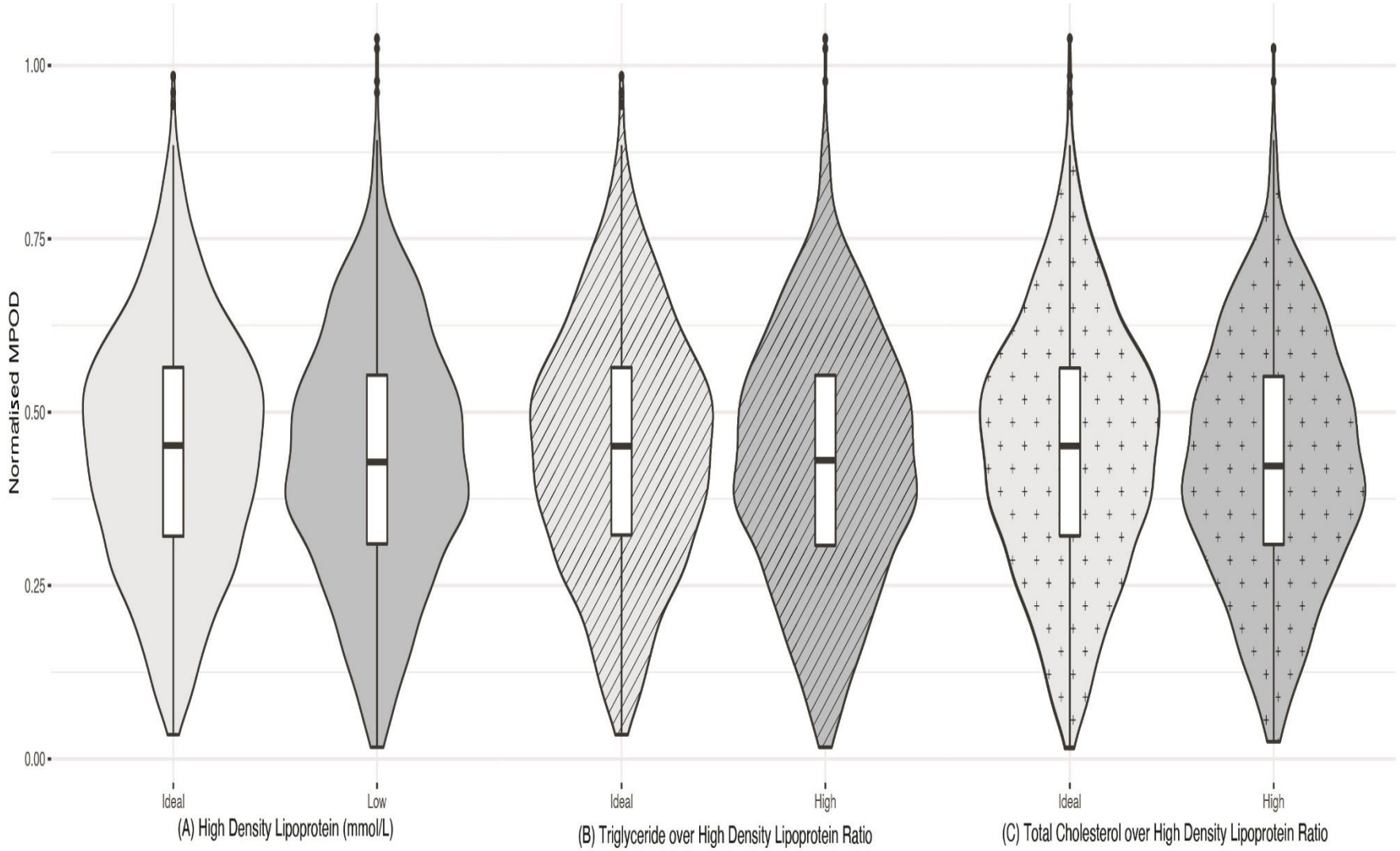

Normalised MPOD
1.00
0.75
0.50
0.25
0.00
Ideal
Low
(A) High Density Lipoprotein (mmol/L)
Ideal
High
(B) Triglyceride over High Density Lipoprotein Ratio
Ideal
High
(C) Total Cholesterol over High Density Lipoprotein Ratio

Figure 8.3: Violin plots of MPOD differences by (A) High Density Lipoprotein (HDL); (B) Triglyceride over High Density Lipoprotein (TG/HDL) Ratio and (C) Total Cholesterol over High Density Lipoprotein (TC/HDL) Ratio (ANOVA).

Mixed linear model effects

A mixed linear model analysis was carried out on normalised MPOD versus demographic, behavioural, anthropometric and plasma biomarker independent variables. Low education, current smoking and elevated WC were significant predictors of MPOD in this regression model (Table 8.3). None of the plasma biomarkers (HDL, TG/HDL, TC/HDL, Vitamin D, CRP or HbA1c) were significantly associated with MPOD in this model (P >0.05 for all variables; Table 8.3).

Table 8.3: Mixed linear model effects; relationship between demographic, behavioural, anthropometric and plasma biomarkers, and MPOD in a normal population.

Parameter	Estimate	Std. error	*T*	(normalised MPOD) *p*
Constant	0.448	0.085	5.297	**0.000**
Sex* (Female)	0.011	0.008	1.284	0.199
Education				
Secondary	0.023	0.010	2.376	**0.018**
Tertiary/higher	0.038	0.010	3.866	**0.000**
Smoking†				
Past	-0.004	0.008	-0.588	0.557
Current	-0.022	0.011	-2.011	**0.044**
WC (cm)	-0.001	0.000	-2.415	**0.016**
HbA1c (%)	0.006	0.013	0.454	0.650
Vitamin D (nmol/L)	-0.000	0.000	-0.728	0.467
CRP ‡ (>3µg/l)	0.000	0.000	0.745	0.456
HDL (mmol/L)	-0.009	0.012	-0.781	0.435
TG/HDL Cholesterol Ratio	-0.001	0.005	-0.161	0.872
TC/HDL Cholesterol Ratio	-0.001	0.005	-0.145	0.885

Hypertension ‡‡ (mmHg) (No)				
Hypertensive	0.008	0.007	1.056	0.291
Hypertensive (not medicated)	0.002	0.010	0.188	0.851
Cataracts‡‡ (No)	0.018	0.014	1.291	0.197

Dependent variable = MPOD sqrt (normalised MPOD).

**Male = reference group; † Never smoked = reference group; ‡ CRP (≤ 3mg/L= reference group); †† No Hypertension = reference group; ‡‡ Yes cataracts = reference group. WC (cms); HbA1c (%); vitamin D (nmol/L), HDL (mmol/L); TG/HDL ratio; TC/HDL ratio – as continuous variables.*

Abbreviations: Std. Error - Standard Error; cms - centimetres; CRP - C-reactive protein; HbA1c- glycated haemoglobin; HDL-high density lipoprotein; µg/l – micrograms per litre; mmHg- millimetres of mercury; nmol/L – nanomoles per litre; WC-waist circumference; TC/HDL- total cholesterol over high density lipoprotein; TG/HDL- triglyceride over high density lipoprotein.

8.5 Discussion

The key finding to emerge from this study is the observation that participants with increased central adiposity, as indicated by an elevated WC, had significantly lower MPOD compared to those with ideal measures. Macular pigment was also lower amongst participants with an altered lipoprotein profile, *i.e.* in participants with raised plasma TG/HDL ratio, raised plasma TC/HDL ratio and low plasma HDL. Additionally, male gender, current smoking and low educational status was associated with lower MPOD, findings which concur with previously reported analyses on a similar cohort.[52] Elevated WC, current smoking and low educational attainment persisted as predictors of low MPOD on mixed linear model analysis, after adjusting for all other covariates.

Consistent with most previous reports,[16, 52, 54, 384] MPOD was, in fact, significantly and negatively associated with all measures of overweight/obesity, including BMI, WC, WHtR, WHpR, (Pearson's r; $p<0.05$ for all). With cut-off levels applied to classify participants into binary categories (obese or non-obese),[527] elevated WC emerged as the main anthropometric variable associated with lower levels of MPOD. Carotenoids, such as lutein and zeaxanthin, are known to accumulate in adipose tissue, and in visceral fat in particular,[54, 219] therefore, distribution of body fat is an important consideration.[67] Higher WC has previously been reported to be the strongest non-dietary predictor of low MPOD,[384] and findings from the Third National Health & Nutrition Examination Survey revealed that participants with higher WC values were increasingly likely to have hypertension, diabetes, dyslipidaemia and the metabolic

syndrome,[555] metabolic correlates independently associated with lower MPOD.[6, 7, 18]

As an indicator of 'abdominal' obesity [384, 555] WC is receiving increased attention as a more clinically relevant measure of adiposity compared with BMI. Intra-abdominal fat may have different metabolic consequences compared with other patterns of fat distribution or overall adiposity, as it is more biologically active. Intra-abdominal fat is associated with inflammation due to the increased production of adipocytokines.[372, 442] The associated inflammation becomes chronic, therefore, obesity is now considered a low-grade inflammatory condition, which in turn leads to an increase in oxidative stress.[69] Chronic inflammation and associated oxidative stress exert a greater demand on antioxidant defences within the body; defences which tend to be lower in the obese,[367] and older adults.[556] Sex differences exist in relation to how body fat is deposited,[449] and there is evidence for gender-related differences in the accumulation of carotenoids.[16, 17] Interestingly, we found that MPOD was 9% lower in males compared with females (p=0.004), possibly explained by the fact that men store more fat in the visceral (abdominal) fat depot,[555] thereby, making these pigments less available to retinal tissue.[68, 326] Thus, lower antioxidant activity may relate not only to lower intake of antioxidants and phytochemical rich foods in older adults (e.g. fruits, vegetables and legumes),[557] reduced activity of endogenous anti-oxidative enzymes,[556,367] but also due to an increased utilisation of these molecules. Collectively, these factors may lead to antioxidant depletion, including lower levels of MP in the eye.[4, 291]

Another interesting finding is the observation that MPOD was significantly lower in participants with raised plasma TG/HDL ratio (>0.87 mmols/L), and in those with raised plasma TC/HDL ratio (>3.5 mmols/L). Although these relationships did not persist on mixed linear model analysis, the lower MPOD levels observed among participants with low plasma HDL corroborates previous findings which identified an association between plasma HDL and MPOD.[462] Dyslipidaemia is often characterised by low levels of HDL, raised TGs and smaller, more atherogenic LDL particles.[160] As carotenoids are bound to circulating lipoproteins, the relation between plasma levels of the various lipoproteins and MPOD is of interest. Evidence suggests that HDL is the major carrier of lutein to the eye.[18] Furthermore, HDL is a known ligand for SR-B1, and the mechanism of xanthophyll uptake by the retina appears to be entirely SR-B1 dependent.[326]

Vitamin D deficiency has repeatedly been associated with ocular disease.[306, 547] Additionally, vitamin D deficiency is common world-wide, particularly so in older adults.[498, 558] Of interest, we recently reported that individuals with Type 2 diabetes who were vitamin D deficient (≤50 nmol/L), had significantly lower MPOD, and that vitamin D was a significant positive predictor of MPOD on multivariate analysis in this study group.[559] The current study, however, suggests that the association between vitamin D status and MPOD does not persist among older adults who are free of ocular pathology. Furthermore, no relationship was observed between MPOD and the inflammatory marker CRP.

This population-based representative study advances previously published work which also utilised TILDA data to explore MPOD,[52] as the analyses herein relied much less on self-reported data. A large number of subjects were excluded from the overall MP analysis (n=2,014), and possible reasons for this included: technical issues, poor fixation and poor VA, among others. While c-HFP is one of the most common methods of measuring MP, it is challenging to perform, particularly so for older adults.[56] Research has shown that some participants have difficulty performing this technique, with dropout rates varying from 10 to 20%.[56, 560] A Chi-square test of independence was performed, however, comparing the centre population (*n*=5,275) and the smaller sample of participants with a valid MPOD assessment (*n*=2,594), and this test confirmed that there was no significant difference in age, sex or education status between the two groups. More importantly, the attenuated number of participants in the current study did not appear to impact on the validity of our findings as we confirmed previously reported analyses on a similar cohort.[52] Despite differences in inclusion criteria, the relationship between education and smoking status and MPOD observed therein and elsewhere was confirmed in our analysis.[52, 384] Some discrepancies did emerge, however. Self-reported measures of high cholesterol, for example, were not associated with MPOD according to the previous analysis,[52] whereas the analysis of direct cholesterol measures taken herein suggest that dyslipidaemia (*i.e.* low plasma HDL, raised TG/HDL, raised TC/HDL) may have implications for the delivery and uptake of MP in the eye. Additionally, while an inverse association between self-reported high blood pressure and MP was also previously reported,[52] we found no association between measured hypertension (treated or untreated) and MPOD on either univariate or mixed linear model analysis.

These direct measurements (systolic and diastolic, mmHg), would appear to outweigh self-reported data.[52] Interestingly, we recently reported that individuals with diabetes and hypertension, had significantly lower MPOD compared to those without,[559] which suggests that when conditions co-exist the redox balance may become increasingly upset, and that oxidative stress may increase considerably in hypertension, especially as a diabetic co-morbidity.[535] Hypertension, however, does not appear to affect or deplete MP, in older adults, who are free of ocular pathology.

A number of variables did not persist as predictors of MPOD on mixed linear analysis, (*i.e.* sex, and plasma lipoproteins), despite significant outcomes on univariate analysis. While gender (male) and lipoprotein (low HDL, raised TG/HDL, raised TC/HDL) status contribute to lower levels of MP, it appears that WC status is a more robust predictor of low MPOD. It is important to note, however, that the small difference in mean MPOD between participants with and without elevated WC suggests that any effect size is likely to be modest and not particularly useful in terms of predictive capacity from a clinical perspective.

Macular pigment was measured using c-HFP, a technique that has been validated in older subjects [335, 560] and found to be reliable.[560] Macular pigment measurements have, however, shown a degree of variability across studies.[59, 335, 384, 561] For example, Stringham et al, [335] measured the density of MP in patients with intermediate AMD, using a similar device and retinal eccentricity (0.5^0), and the mean optical density was (0.37±0.24). Subjects in CAREDS had an average MP level of (0.36±0.22) right eye and (0.34±0.21) left eye, and these participants were of a similar age.[384] We

acknowledge that the mean MPOD in the current study was notably lower than previously reported.[335, 384] While, we found no correlation between age and MPOD in the current study, our findings suggest that older Irish adults have lower MP levels on average. It is worth noting that the prevalence of conditions which might affect MPOD levels is relatively high in Ireland, including AMD,[562] glaucoma,[563] and Type 2 diabetes.[130] The lower levels of MP observed in the current study, may, therefore, reflect the combined influence of inadequate dietary intake and increased utilisation of these phytonutrients.[556] Given the variety of mechanisms which may contribute to macular pigment depletion, and the ocular risks associated with such depletion in this age group, the importance of diet cannot be over-emphasised. The provision of coloured fruit and vegetables which are rich in a broad range of antioxidants including the target carotenoids lutein and zeaxanthin and/or supplementation with these nutrients represents an obvious recommendation, given the putative protection that this pigment confers against age-related vision loss. These recommendations should now be subject to further research.

8.6 Limitations

Limitations of the current study include the failure to assess dietary intake or plasma levels of the carotenoids lutein and zeaxanthin as MPOD is influenced by both intake and individual efficacy of absorption.[70] It is also worth noting that the high variability and lower levels of MPOD observed across different people may also be subject to genetic variation [72] a factor that is not accounted for in our analysis. Information on supplement usage was also not assessed, which would have added to the interpretation of our findings, particularly those in relation to vitamin D and MPOD.

Despite these limitations, there were a number of strengths to the current analysis. Plasma lipoproteins and inflammatory markers were analysed and blood pressure was measured, an advance on previously used self-reported data.[52] Participants with comorbidities, AMD, glaucoma and diabetes, were eliminated from the current analysis and HbA1c was used to help identify undiagnosed and pre-diabetes cases. The use of measured rather than self-reported anthropometric data for WC, WHtR and WHpR is a further strength of the study. Finally, the representative study design of the TILDA study, the unique sampling method which includes both males and females, and the detailed exclusion criteria, we believe that our findings and the conclusions drawn from our findings concerning MPOD are representative of the older Irish population.

8.7 Conclusion

In conclusion, we report that elevated WC and dyslipidaemia may have important implications for the storage, delivery and uptake of lutein and zeaxanthin in the eye. Determinants of MPOD (*i.e*. education status, tobacco use, WC), remain statistically significant following mixed linear model analysis. Although no strong predictor of MPOD status emerged in this analysis, the capacity of more commonly-measured surrogate biomarkers to help identify people at risk of low MP merits further consideration. Given the marginal statistical significance of many of our findings, further research is necessary to refine our understanding of the observed relationships as MP is not routinely measured in clinical practice.

9. SUMMARY, CONCLUSIONS AND DIRECTION FOR FUTURE WORK

9.1 Summary and conclusions

This work was designed to explore the optical density of MP in patients with diabetes mellitus and to compare their levels with those of normal healthy controls. The findings reported and the conclusions drawn herein are based on the outcomes of two cross-sectional, case-controlled studies, one of which investigated MPOD in participants with Type 1 and Type 2 diabetes in a hospital setting; and the other, which explored MPOD amongst Type 2 diabetes participants in TILDA, a large, randomly-selected population representative of older adults in Ireland. Although the optical density of MP can be measured *in vivo* it is not routinely measured in clinical practice. Therefore, as part of this research, surrogate biomarkers for the prediction of MPOD in patients at high risk of MPOD depletion (*i.e.* Type 2 diabetes and older adults) were investigated and identified in the TILDA cohort. A literature review of the evidence describing macular carotenoid depletion in diabetes (particularly Type 2 diabetes) was also performed, to identify the causal mechanisms by which diabetes mellitus and its metabolic perturbations contribute to lower MP levels. The conclusions and future research proposed as a result of the outcomes of this work are as follows.

Macular pigment is lower in Type 2 diabetes

Consistent with previous investigators who have explored the relationship between MPOD and diabetes, [6, 9] this research provides further evidence that low MP may indeed be a feature of diabetes. Furthermore, the current work demonstrates that MPOD is significantly lower in those with Type 2 diabetes participants compared to

those with Type 1; this represents an entirely novel finding. Overall, the behavioural (smoking), clinical (blood-pressure, presence of cataracts), anthropometric (body weight, BMI, WHtR) and plasma (lipoproteins, inflammatory markers) parameters of participants with Type 2 diabetes were poorer than those observed in non-diabetic controls. Hyperglycaemia and other anthropometric, metabolic and clinical correlates associated with diabetes may, therefore, constitute readily measurable proxies for the presence of chronic oxidative stress, inflammation and other pathological processes which underlie many of the functional alterations observed in diabetic retinopathy, and which also appear to be responsible for MPOD depletion in diabetes.

Surrogate biomarkers for the prediction of patients at risk of low macular pigment in Type 2 diabetes.

Tobacco use and the presence of cataract were significantly associated with lower MPOD levels amongst participants with diabetes. Metabolic comorbidities characteristic of Type 2 diabetes (*i.e.* overweight/obesity, dyslipidaemia and hypertension) were also significantly negatively associated with MPOD. The combined effect of increased lutein/zeaxanthin sequestration by excess adipose tissue, increased inflammation and elevated oxidative stress associated with overweight/obesity (*i.e.* BMI, WHtR, WC), appear to explain, at least in part, the lower MPOD levels observed in these Type 2 diabetes participants. Unfavourable lipoprotein status (*i.e.* raised TG/HDL) may also contribute to lower MPOD, as MP carotenoids are primarily transported by HDL in plasma,[18, 71] and mechanisms governing their retinal capture and/or stabilisation in the retina are also subject to HDL influence.[71] Furthermore, hypertension, especially as a co-morbidity of

diabetes, appears to further deplete MPOD levels due to its role in elevated oxidative stress.[535] The borderline statistical significance of these findings, however, means that more work needs to be done on larger, purposively selected study populations to verify and refine our understanding of how these factors affect MPOD levels.

Vitamin D emerged as a positive predictor of MPOD in participants with Type 2 diabetes which is again, a novel finding. In addition, MP levels were significantly lower in participants with Type 2 diabetes who were deficient in vitamin D (*i.e.* ≤ 50nmol/L). Vitamin D status does not appear to be associated with MPOD in older adults who are free of ocular pathology (*i.e.* AMD, glaucoma, diabetes/pre-diabetes). These findings suggest that the chronic low-grade inflammation and oxidative stress associated with Type 2 diabetes may indeed explain the lower MPOD levels observed in those with this condition and that the low vitamin D status associated with these pathological processes may itself be used as a predictor of low MPOD in patients with diabetes (Type 2). Findings from the DiVFuSS trial [21] are promising and appear to suggest that supplementation with a compound which specifically targets both inflammation and oxidative stress (*i.e.* vitamin D and carotenoids, lutein/zeaxanthin and *meso*-zeaxanthin) may be beneficial in protecting against the mechanisms underlying diabetic retinopathy.[21] Furthermore, in line with previous research,[307] a dilated eye examination might be recommended for diabetes patients whose low plasma vitamin D levels indicate an elevated risk of diabetic retinopathy. Further research is, however, needed to articulate the links between inflammatory status, disease progression and the ameliorative effects of supplementation with carotenoids and other vitamins (*i.e.* vitamin D) amongst those with diabetes.

Current findings suggest that the exogenous antioxidants lutein, zeaxanthin and *meso*-zeaxanthin, become depleted in diabetes. Although no strong predictor of MPOD depletion emerged, the capacity of more commonly used everyday measures to help identify patients at risk of low MPOD in diabetes, such as anthropometric status (WC, WHtR) and plasma biomarkers (TG/HDL, vitamin D) merits further consideration. Further research should now be undertaken to refine our understanding of these observed relationships.

Causal mechanisms putatively associated with lower macular pigment in diabetes mellitus.

A review of the evidence pertaining to MP levels in diabetes mellitus suggests that MP is lower in diabetes **(**Type 2 diabetes in particular). Candidate causal mechanisms to explain the lower MP levels reported include oxidative stress, inflammation, overweight/obesity and dyslipidaemia. In summary, hyperglycaemia, oxidative stress and changes in redox homeostasis are fundamental events in the pathogenesis of diabetic retinopathy. The body's natural defence against oxidative damage is neutralisation by endogenous antioxidants (enzymatic and non-enzymatic), supported by exogenous antioxidants (*i.e.* vitamin C, vitamin E, carotenoids lutein, zeaxanthin and *meso*-zeaxanthin). While oxidative stress is increased in diabetes, the activities of the antioxidant defence system are concomitantly diminished. In addition, inflammation is involved in the development of diabetic complications. Inflammatory processes underlie many of the functional changes in retinal vasculature observed histologically in early diabetic retinopathy. Oxidative stress triggers inflammatory responses and inflammation also enhances the production of ROS. Locally, the

occurrence of these metabolic changes can lead to MPOD depletion in the eye. Adipose tissue (more common in Type 2 diabetes) is another source of inflammation and also a major body store for carotenoids, lutein and zeaxanthin. Competitive storage by increased visceral fat may not only compete for retinal uptake but also independently increase oxidative stress and inflammation, thereby, reducing antioxidant capacity and MP levels in the eye. Finally, reduced insulin action in Type 2 diabetes may also increase the risk of dyslipidaemia. The characteristic lipid profile of an individual with diabetes (*i.e.* increased TGs and low HDL) may have important implications for the delivery and uptake of MP in the eye.

Impaired defence against ROS at the retina may not only be attributable to the increased utilisation of these antioxidants in diabetes (via chronic inflammation and oxidative stress) but may also arise as a result of increased adiposity (visceral fat in particular) and dyslipidaemia (*i.e.* low HDL, raised TGs), metabolic correlates which may adversely affect MP by compromising the availability, transport, and assimilation of these dietary carotenoids in the eye. Furthermore, lower intake of foods rich in carotenoids and other nutrients (*i.e.* fruit, vegetables, legumes), is common in Type 2 diabetes. Overall, dietary advice focused on enhanced antioxidant supply and the optimal provision of anti-inflammatory nutrients (e.g. vitamin D, omega-3 essential fatty acids), along with lifestyle modifications such as increased physical activity to better manage bodyweight and plasma lipids, may be an effective prophylactic means to control oxidative stress/inflammation in diabetic eye disease and potentially slow the progression of diabetic retinopathy.

Surrogate biomarkers for the prediction of patients at risk of low macular pigment in older Irish adults.

Finally, given the possibility that higher levels of MP may be beneficial for vision and ocular health, and that MPOD is not routinely measured in clinical practice, a range of behavioural, anthropometric, clinical and plasma measures were also explored as possible predictors of low MPOD amongst a cohort of non-diabetic older adults. Several findings emerged from this investigation. Of note, low educational status, current smoking and elevated WC were all significant predictors of lower MPOD in this non-diabetic cohort. That an elevated WC (>102cm in males, >88cm in females) was independently associated with lower MPOD levels is particularly interesting, given that participants with higher WC values are also more likely to have hypertension, diabetes, dyslipidaemia and the metabolic syndrome,[555] metabolic correlates independently associated with lower MPOD. [6, 7, 18] This lends credence to the theory that central adipose sequestration of carotenoids plays a role in MPOD depletion, and adds further support to the recommendation that WC be a routine measure in identifying patients at risk of low MPOD. However, further research is needed in this area as the difference in MPOD (0.013 OD), while statistically significant, was not at a level deemed clinically significant.

Macular pigment was significantly lower amongst non-diabetic participants with low plasma HDL, raised plasma TG/HDL ratio, and raised TC/HDL ratio. Of interest, dyslipidaemia can often precede the overt appearance of Type 2 diabetes by several years.[171] These findings suggest that the characteristic lipid profile of individuals who are overweight or obese, or who have emerging metabolic disease or established

Type 2 diabetes,[158] (*i.e.* raised TGs and low HDL), may indeed have important implications for MP levels in the eye. While plasma lipoprotein biomarkers were not predictive of low MPOD on regression analysis, these preliminary findings warrant further investigation to fully elucidate any relationships which might exist.

Eye-care practitioners are well-positioned to help motivate patients towards dietary modification (for example enhanced carotenoid and vitamin D intake) and lifestyle changes (such as increased physical activity). Notably, formal educational status was a positive predictor of MPOD amongst older subjects. It is well known that educational status is a marker for overall socioeconomic status (SES),[564] and that lower SES is associated with a constellation of risk factors thought to mitigate the accumulation of carotenoids at the macula, (*i.e.* tobacco use,[65] overweight/obesity,[16, 17, 54] dyslipidaemia,[18] oxidative stress,[552] and inflammation).[553] Furthermore, research has shown that people who are less educated with lower incomes consume more energy-dense, micronutrient-dilute food, compared with their higher SES counterparts, who have higher fruit and vegetable intake.[565] Current smoking emerged as a significant predictor of low MPOD in both Type 2 diabetes participants and amongst older, non-diabetic adults. Apart from its association with lower overall antioxidant intakes and increased oxidative stress and antioxidant utilisation, smoking is also a significant, yet preventable, direct cause of trauma to the eye which can lead to blindness. Given the emerging evidence that MP is important for vision and ocular health,[4] and that smoking is an established modifiable risk factor for AMD,[49, 62] more effort is needed to increase patient awareness of the harmful effects of smoking on the eye. Clinical practitioners can play an important role in educating

patients on the importance of carotenoid intake (*i.e.* fruit, vegetables, legumes) and of smoking cessation, lifestyle changes which may help to maintain healthy eyes in the long-term.

Given the ready availability of such data, the use of commonly measured proxies to help identify people at risk of low MP is worth exploring. The marginal statistical significance of many of our findings in this area means that further research is necessary to refine our understanding of these observed relationships. In time, this may contribute to the establishment of a checklist to be used in the screening and identification of individuals at high risk of MPOD depletion and its consequent ocular pathologies.

9.2 Strengths and limitations of the current research

The different presenting features of Type 1 and Type 2 diabetes and the interplay of their disease components with MPOD were analysed and compared with non-diabetic controls in the initial study. There were several strengths to this analysis. The MPOD was measured using c-HFP method, dietary intake of carotenoids was quantified, plasma lipids (TC, HDL, TG, and LDL) and HbA1c were measured, to explore the influence of potential explanatory factors on central MPOD in diabetes (Type 1 and Type 2). There were, however, a number of limitations which should also be recognised. While c-HFP technique is considered the gold standard psychophysical method for measuring MPOD, the clinical densitometer can only take two measurements, centrally and peripherally, therefore, providing no information on the spatial distribution of MP. Although, dietary intake of lutein and zeaxanthin was

quantified, the FFQ used in this study was designed for an American population (among Irish participants), which was not ideal. The lack of plasma carotenoid analysis was also a drawback. Body mass index was used to assess overweight/obesity, however, this measure alone does not provide a precise indicator of adiposity, visceral fat in particularly (more commonly found in Type 2 diabetes). Therefore, future studies investigating the link between MPOD and obesity should encompass more refined methods of body fat assessment including WC, WHtR and WHpR. Inflammatory markers (*i.e.* vitamin D and CRP) were also not analysed as part of this study. Finally, small patient numbers in some of the sub-group analyses may have limited our ability to detect statistical effects.

Findings from this study, however, helped inform the type of analysis performed in the TILDA study. There were a number of strengths to the TILDA study. The design of the study itself was a particular strength. Diabetes participants and non-diabetic controls were drawn from a large randomly selected sample, representative of the Irish population aged 50 and over. Plasma lipoproteins including the various lipoprotein fractions (TC/HDL, TG/HDL, non-HDL) and inflammatory markers (vitamin D, CRP) were analysed and blood pressure was measured. The additional use of HbA1c helped identify undiagnosed and pre-diabetes cases. Overall, the detailed exclusion criteria used was a key strength of the study. Anthropometric measures such as WHtR, WHpR and WC, were also used to analyse overweight/obesity, an advance on previous analysis which used BMI only. There were, however, also a number of weaknesses to the study, which are worth noting. Firstly, the use of self-reported data was not ideal, particularly concerning diagnosis of diabetes (Type 1 vs Type 2), duration of disease

and the presence of cataract (all self-reported). Customised-HFP was used to measure MPOD, which requires significant patient training to produce meaningful results, particularly for older participants. A large number of subjects had to be excluded from the current study (n=2,014) due to their inability to successfully complete MPOD measurements. In addition, small participant numbers in some of the diabetic sub-group analyses may have limited statistical power to detect associations (*i.e.* Type II error). Furthermore, dietary intake of lutein and zeaxanthin and plasma analysis of carotenoids were not assessed, therefore, it was not possible to control for potential confounding from the variable intake of lutein and zeaxanthin.

Finally, the review paper provided an in-depth analysis of the causal mechanisms and metabolic perturbations putatively associated with lower MP in diabetes (Type 2 diabetes in particular).This article provided a holistic evaluation of diet (and/or supplementation) and lifestyle in relation to MPOD and diabetes and the latest available evidence was explored. Some limitations which prevailed, however, included small sample sizes in the reviewed literature and the merging of Type 1 and Type 2 diabetes patients in statistical analyses. Additionally, the complex interplay of diabetes and its metabolic correlates (adiposity, dyslipidaemia, oxidative stress and inflammation) with MPOD status represented a particular challenge.

9.3 Directions for future work

Macular pigment has a unique concentration within the retina and is biologically relevant to the protection of the eye.[44, 45] While existing evidence suggests that MP is protective against the onset and progression of diabetic retinopathy based on animal

studies,[14, 20, 186] and one RCT,[21] the potential protective effects of MP against diabetic retinopathy in humans need be clarified, particularly in cases where MP levels are low. Therefore, the findings of this current work should now prompt and inform the development of a well-designed, placebo-controlled clinical trial to help us further understand the relationship which exists between MPOD and diabetes, and the implication of this relationship for ocular health.

Future studies should measure carotenoid intake and MPOD levels in a range of patients with diabetes (Type 1 & Type 2), at various stages of the disease (*i.e.* no retinopathy, non-proliferative, proliferative, with or without diabetic macular oedema), and compare their clinical outcomes, carotenoid intake and MPOD levels with those of individuals not afflicted with the condition. Disease progression and macular health should be monitored prospectively (ideally over 2 years or more). As dietary estimation of carotenoids and antioxidants is complex and challenging, this work should also be extended to embrace placebo-controlled supplementation trials with both ocular carotenoids in isolation (lutein, zeaxanthin and *meso*-zeaxanthin alone) or as part of a multi-component antioxidant/anti-inflammatory preparation. Clinical outcome measures should include MPOD augmentation, structural parameters of the macula (as assessed with OCT) and disease progression.

Insulin resistance, impaired glucose tolerance and Type 2 diabetes can exist for many years before retinal signs become evident in diabetes, therefore, it is important that both the clinical (HbA1c, lipids, BP and inflammatory markers) and histological (structural) aspects of the condition are recognised and monitored. There is merit in

exploring the association between MPOD and more commonly assessed parameters such as behavioural (tobacco use), anthropometric (WC, WHtR), plasma (HDL, TG/HDL, vitamin D and CRP) and clinical (blood pressure, presence of cataracts) measures. This could help to determine whether MPOD levels and specifically the risk of low MPOD status, can be predicted using these data. Also, whether clinical interventions designed to alter these parameters and the course of the disease itself can also elicit more favourable MPOD status and ocular health outcomes.

Clinical health benefits may be realised through the early identification of MPOD deficits in diabetes patients and through the implementation of appropriate interventions (supplementation with carotenoids and other nutrients) which may help to prevent or slow the progression of diabetic retinopathy. Importantly, a combination of lifestyle behaviours such as the avoidance of smoking, physical activity, and the adoption of a healthy dietary pattern (*i.e.* increased fruit and vegetable intake) may not only lower the prevalence of Type 2 diabetes but may also protect against the cumulative pathological mechanisms which precipitate diabetic retinopathy.

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APPENDICES

Appendix 1: Macular Pigment and Diabetes Consent Form

Macular Pigment Optical Density Study and Diabetes

We are currently recruiting for an observational study looking at the levels of macular pigment in a diabetes population at the Mater Hospital, in conjunction with the Dublin Institute of Technology. The main aim is to examine whether the levels of macular pigment are correlated with diabetes, and also the level of diabetic retinopathy that each individual may have, and assess whether changes in the levels of these pigments affect visual acuity. Participation in this study is entirely voluntary and non-participation does not affect your current treatment at the Mater Hospital. All results will be examined anonymously.

Participation involves photographing the back of the eye with a special non-invasive instrument which measures the levels of macular pigment. As these levels can be affected by dietary intake, we will also conduct a food intake questionnaire to control for this. We envisage performing these tests and questionnaires at the time of your screening/OPD clinic visit. Should you wish to participate in this important research study please sign on the dotted line below as consent.

Paul Connell

Consultant Vitreoretinal Surgeon Mater Hospital. Date: ……

Appendix 2: Diabetes & Macular Pigment Study - Data Capture Form

EXCLUSION CRITERIA:

- Ocular diseases other than non-proliferative diabetic retinopathy
- Previous ocular surgery or ocular trauma
- Best corrected vision < 6/18
- Taking MP supplements (e.g. Macushield, Ocuvite Lutein, Vitalux, Vision Ace, Lutein Omega 30, etc)

Date (D/M/Y): _____

Location: _____

Name: _____

MRN: _____

Sex: M / F DOB (D/M/Y): _____

Race: _____

Current smoker: Y / N Previous smoker: Y / N

BMI (kg/m^2): _____

Type of diabetes: None: ____ Type 1: ____ Type 2: _____	Duration of diabetes (years):

Study group: ____ Non-diabetic (control) ____ Diabetes, no DR	Right visual acuity:

____ Diabetes, non-proliferative DR Study Eye: Right eye: ____ Left eye: ____	Left visual acuity:

Previous laser treatments for DR: Right eye: Y / N Date: _____ Left eye: Y / N Date: _____	Medications for diabetes: ____ Insulin ____ Oral hypoglycaemics Multivitamin supplements? Y / N Specify:_____

Systemic comorbidities:

Most recent blood data recording: Date (D/M/Y):

HbA1C (%):	
Triglycerides (mmol/L):	
HDL cholesterol (mmol/L):	
LDL cholesterol (mmol/L):	
Total cholesterol (mmol/L):	

MPOD Right: _____ SD: _____ MPOD Left: _____SD:

Appendix 3: Lutein and Zeaxanthin FFQ:

LZQ™: How often do you eat the following foods?

Food Categories	Food items and a prompt for clarification			Times Per Day	Times Per Week	Times Per Month
BREADS	Cornmeal, yellow	1	Tbsp.	to	to	to
	Cornmeal, white	1	Tbsp.	to	to	to
	Cornbread Muffin	1	each	to	to	to
	Corn Tortilla, 6"	1	each	to	to	to
	Bread, roll, bun, bagel	1	slice	to	to	to
CEREALS	Apple Jacks™	1	cup	to	to	to
	Cap'n Crunch ™	1	cup	to	to	to
	Corn Chex ™	1	cup	to	to	to
	Corn Flakes	1	cup	to	to	to
	Corn Pops	1	cup	to	to	to
	Crispix ™	1	cup	to	to	to
	Froot Loops ™	1	cup	to	to	to
	Frosted Flakes	1	cup	to	to	to
	Life™	1	cup	to	to	to
	Reese's Puffs ™	1	cup	to	to	to
CONDIMENTS	Fat Free Mayonnaise	1	Tbsp.	to	to	to
	Regular Mayonnaise	1	Tbsp.	to	to	to
SAUCES	Sauce, Ready-To-Serve, Pepper or Hot	1	Tsp.	to	to	to
	Sauce, Salsa, Ready-To-Serve	0.5	cup	to	to	to
FRUITS	Apple (with skin), medium	1	each	to	to	to
	Apricots see prompt.	0.3	cup	to	to	to
	Cantaloupe	1	cup	to	to	to
	Red Grapes	1	cup	to	to	to
	Green Grapes	1	cup	to	to	to
	Kiwi	1	each	to	to	to
	Mango, medium	1	each	to	to	to
	Honeydew	1	cup	to	to	to
	Nectarine, medium	1	each	to	to	to
	Orange Juice	1	cup	to	to	to
	Peaches (canned)	1	cup	to	to	to
	Peach (fresh), medium	1	each	to	to	to
	Watermelon	1	cup	to	to	to
NUTS	Pistachios	1	oz.	to	to	to
PASTA	Macaroni and Cheese	1	cup	to	to	to
	Egg Noodles	1	cup	to	to	to
	Spinach Egg Noodles	1	cup	to	to	to
EGGS	Egg, including yolk, large	1	each	to	to	to
SNACKS	Chex Mix™	1	cup	to	to	to
	Cheetos™	1	oz.	to	to	to
	Fritos™	1	oz.	to	to	to
	Popcorn	1	oz.	to	to	to
	Tortilla Chips	1	oz.	to	to	to
VEGETABLES	Artichoke Quarters (bottled or canned)	1	cup	to	to	to
	Asparagus (cooked)	1	cup	to	to	to
	Green Beans (cooked)	1	cup	to	to	to
	Broccoli (raw or cooked)	1	cup	to	to	to
	Brussels Sprouts (cooked)	1	cup	to	to	to
	Red Cabbage (cooked)	1	cup	to	to	to
	Cilantro (raw),	1	Tbsp.	to	to	to
	Yellow corn	1	cup	to	to	to
	Cucumber (with skin), medium	1	each	to	to	to
	Endive (raw)	1	cup	to	to	to
	Kale	1	cup	to	to	to
	Iceberg Lettuce	1	cup	to	to	to
	Avocado (California)	1	each	to	to	to
	Romaine Lettuce	1	cup	to	to	to
	Lima Beans	1	cup	to	to	to
	Green Olives	6	olives	to	to	to
	Spring Onions, Scallions (raw)	1	Tbsp.	to	to	to
	Spring Onions, Scallions (cooked in oil)	1	Tbsp.	to	to	to
	Parsley (raw)	1	Tbsp.	to	to	to
	Green Peppers	1	cup	to	to	to
	Orange Peppers	1	cup	to	to	to
	Red Peppers	1	cup	to	to	to
	Yellow Peppers	1	cup	to	to	to
	Spinach (cooked)	1	cup	to	to	to
	Spinach (raw)	1	cup	to	to	to
	Acorn Squash	1	cup	to	to	to
	Butternut Squash	1	cup	to	to	to
	Yellow Squash	1	cup	to	to	to
	Zucchini	1	cup	to	to	to

Appendix 4: TILDA Documentation

TILDA. (2019). *The Irish Longitudinal study on Ageing (TILDA) Wave 1, 2009-2011*. [dataset]. Version 1.9. Irish Social Science Data Archive. SN:0053-01. www.ucd.ie/issda/data/tilda/wave1

Tilda Data Documentation: https://tilda.tcd.ie/data/documentation/

TILDA Release Guide v4.1

https://www.ucd.ie/issda/t4media/0053-00_TILDA_Release_Guide_v4.1.pdf

The Design of the Irish Longitudinal Study on Ageing

https://tilda.tcd.ie/publications/reports/pdf/Report_DesignReport.pdf

Methodology:https://tilda.tcd.ie/publications/reports/pdf/w1-key-findings-report/Chapter11.pdf

Fifty plus in Ireland 2011;First results from the Irish Longitudinal Study on Ageing (TILDA): https://tilda.tcd.ie/publications/reports/pdf/w1-key-findings report/Tilda_Master_First_Findings_Report.pdf

Computer Aided Personal Interview - TILDA

https://www.ucd.ie/issda/t4media/005301_TILDA_Wave1_CAPI_Questionnaire.pdf

Wave 1 Self Completion Questionnaire

https://tilda.tcd.ie/data/documentation/doc/wave1/Wave%201%20Self%20Completion%20Questionnaire.pdf

TILDA Wave 1 Derived Variables Codebook

https://www.ucd.ie/issda/t4media/0053-01_TILDA_Wave1_Derived_Variables_Codebook_v1.9.pdf